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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5): **09/763210**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
US99/18515	**13 August 1999 (13.08.99)**	**14 August 1998 (14.08.98)**

TITLE OF INVENTION
ADENOVIRUS FORMULATIONS FOR GENE THERAPY

APPLICANT(S) FOR DO/EO/US
SHIH, Shian-Jiun; MCGLENNON, Karen, R; MOODY, Dewey

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)), and Power of Attorney. **(unexecuted)**
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [ ] An information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information: Print EFS

CERTIFICATION UNDER 37 CFR 1.10

"Express Mail" MailingNumber EL620187164US Date of Deposit 14 Feb. 2001

I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Box PCT, Assistant Commissioner for Patents, Washington, D.C. 20231, Attn. EO/US

Debra L. Wetherell (Type or print name of person mailing paper)

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U.S. APPLICATION NO. (If Known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/763210	US99/18515	A2922A US

17.		CALCULATIONS	PTO use only
[X]	The following fees are submitted: Basic National Fee (37 CFR 1.492(a)(1)-(5): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445 (a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO..... **$970.00** International preliminary examination fee paid to USPTO (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... **$840.00** International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2) paid to USPTO..... **$690.00** International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... **$670.00** International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)..... **$ 96.00**		
	ENTER APPROPRIATE BASIC FEE AMOUNT =	**$690.00**	
	Surcharge of $130.00 for furnishing the oath or declaration later than [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	33 - 20 =	13	X $ 18.00	$ 234.00	
Independent Claims	2 - 3 =	0	X $ 80.00	$	
Multiple dependent claim(s) (if applicable)			+ $270.00	$	
			TOTAL OF ABOVE CALCULATIONS =	$ 924.00	
	Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).			$	
			SUBTOTAL =	$	
	Processing fee of $130.00 for furnishing the English translation later the [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.492(f)).		+	$	
			TOTAL NATIONAL FEE =	$	
	Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +			$	
			TOTAL FEES ENCLOSED =	$ 924.00	
				Amount to be refunded	$
				charged	$924.00

a. [ ] A check in the amount of $_____ to cover the above fee is enclosed.

b. [X] Please charge my Deposit Account No. 18-1982 in the amount of $924.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-1982. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Customer No. 005487

Signature Barbara E. Kurys

Name **Barbara E. Kurys**

Registration Number #34,650

Date 14 February 2001

ADENOVIRUS FORMULATIONS FOR GENE THERAPY

This application claims the benefit of co-pending provisional application 60/096,600, which was filed August 14, 1998, the disclosure of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to a formulation for the preservation of viral particles and viral vectors, which is directly injectable into an organism. It relates more particularly to a formulation for a recombinant adenovirus vector that optimally enhances the vector titer, or stabilizes the vector at refrigerator or room temperature, or both. The invention relates to compositions comprising a recombinant adenovirus vector and a concentration of human serum albumin (HSA) effective to stabilize the adenovirus vector at a temperature above the freezing point of water or to enhance a titer of the adenovirus vector compared to a titer in the absence of HSA, or both, in an aqueous buffer.

BACKGROUND OF THE INVENTION

In cell and gene therapy, as well as in blood transfusion or bone marrow transplantation, one of the principal problems encountered is that of the preservation of biological material. It is indeed important to be able to preserve the biological material, under good conditions of viability, for a sufficiently long period of time compatible with industrial scale production and storage and also to make it possible to carry out certain tests. The most commonly used method of preservation consists of freezing the material. However, during the freezing and thawing of biological material, loss of viability and/or infectivity may occur.

Human serum albumin is a non-glycosylated monomeric protein of 585 amino acids, with a molecular weight of 66 kD. Its globular structure is maintained by 17 disulphide bridges, which create a sequential series of 9 double loops (Brown, J.R., 1977). "Albumin structure, function and uses", Rosenoer, V.M. *et al.* (eds.), Pergamon Press, Oxford, pp. 27-51). The genes encoding HSA are known to be highly polymorphic, and more than 30 apparently different genetic variants have been identified by electrophoretic analysis under varied conditions (Weitkamp, L.R. *et al.*, 1973. Ann. Hum. Genet., 37:219-226). The HSA gene comprises 15 exons and 14 introns comprising 16,961 nucleotides, from the supposed "capping" site up to the first site of addition of poly(A).

Human albumin is synthesized in the hepatocytes of the liver, and then secreted into the peripheral blood. This synthesis leads, in a first instance, to a precursor, prepro-HSA, which contains a signal sequence of 18 amino acids directing the nascent polypeptide in the secretory pathway.

HSA is the most abundant blood protein, with a concentration of about 40 grams per liter of

serum. Therefore, there are about 160 grams of circulating albumin in the human body at any one time. The most important role of HSA is to maintain a normal osmolarity of the blood. It also has an exceptional binding capacity for various substances and plays a role both in the endogenous transport of hydrophobic molecules, such as steroids and bile salts, and in that of different therapeutic substances, which may thus be transported to their respective sites of action. Furthermore, HSA has been recently implicated in the breakdown of the prostaglandins.

HSA has been previously shown to stabilize solutions of proteins, including protein antigens, and small organic molecules, such as hemin (Paige, A.G. *et al.*, 1995. Pharmaceutical Res., 12:1883-1888; Chang, A.-C. and R.K. Gupta, J., 1996. Pharm. Sci., 85:129-132; Niemeijer, N. R.. *et al.*, 1996. Ann. Allergy Asthma Immunol., 76:535-540; and Cannon, J.B. *et al.*, 1995. PDA:J. Pharm. Sci. & Tech., 49:77-82.) HSA is purified from material source of human serum, or can be obtained from genetic engineering, whether by fermentation of recombinant cells (bacteria, yeast, or mammalian cells), or by expression in transgenic animals, particularly from mammary tissues. HSA has also been used to preserve biological materials for freezing (WO97/33975). However, this use has not been described for room temperature preservation and storage of adenoviruses.

Preservation of adenovirus for clinical use has become a significant issue as clinical trials progress to phase II and, ultimately, regulatory approval. Presently, adenovirus formulations, such as formulation 1 (Example 1, *infra*), require storage at -70°C to remain stable. The requirement to keep viral vector preparations at these temperatures necessitates acquisition by clinicians of freezers that maintain -70°C temperatures. Another complication arises during shipment of the vectors from the manufacturing site to the clinic. Until the present invention, there was little hope and even lower expectation that adenovirus vectors could be formulated for stable storage, with preservation of infectivity, at standard freezer (-20°C), refrigerator (4°C), or room (20°C) temperatures.

As demonstrated in the Examples, *infra*, the present invention addresses and overcomes these deficiencies in the art, and unexpectedly provides, for the first time, formulations that provide for long term stability of adenovirus vectors at such higher temperatures than have been achieved to date.

The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

SUMMARY OF THE INVENTION

The present invention advantageously provides a formulation for the preservation and/or storage of a recombinant virus vector, particularly and preferably an adenovirus vector, that optimally enhances the vector titer, or stabilizes the vector at refrigerator or room temperature, or both. Thus, in a first embodiment, the invention provides a composition comprising a recombinant adenovirus vector and a

concentration of serum albumin effective to stabilize the adenovirus vector at a temperature above the freezing point of water or to enhance a titer of the adenovirus vector compared to a titer in the absence of serum albumin, or both, in an aqueous buffer at a pH effective to stabilize the adenovirus vector. In a specific embodiment, the serum albumin is human serum albumin (HSA). In a specific embodiment, the concentration of HSA is from about 0.01% to about 25% (w/v). Preferably, the concentration of HSA is from about 0.1% to about 15%. More preferably, the concentration of HSA is from about 1% to about 10%. Most preferably, the concentration of HSA is about 5%. HSA can be purified from natural sources, or, more preferably, obtained by genetic engineering.

The advantage of such a formulation stems from the fact that the solution is available for administration immediately after removal from the storage temperature, without any further manipulation being necessary. It then becomes possible to carry out the removal from storage conditions directly in the clinic, thereby reducing the time between storage and use, which also makes it possible to remain constantly in a sterile formulation and therefore to reduce to a minimum the risks of external contamination.

In a further embodiment of the invention, the pH of the composition is greater than or equal to 5.0 and less than or equal to 9.0. Preferably, the pH is greater than 7.5. Thus, the pH can be 7.6, 7.7, 7.8, or 7.9. More preferably, the pH is greater than 8.0, *e.g.*, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, or 8.9. In a specific embodiment, the pH is 8.2. In another specific embodiment, the pH is 8.4. Preferably, when the pH of a composition of the invention is greater than 8.0, the concentration of HSA is 1% to 10%; more preferably 5%. In a specific embodiment, when the pH is 8.2, the concentration of HSA is 5%. In another specific embodiment, when the pH is 8.4, the concentration of HSA is 5%.

The aqueous buffer of the invention comprises a physiological buffer, such as, but by no means, limited to phosphate buffer, Tris-HCl buffer, Hepes buffer, and the like. A suitable buffer for use according to the invention is pharmaceutically acceptable, *i.e.*, compatible with *in vivo* delivery. In an embodiment exemplified *infra*, the buffer is a Tris-HCl buffer. The buffer is set at a pH that stabilized adenovirus vectors. An aqueous buffer for use in the invention comprises salts, *e.g.*, calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), and sodium chloride (NaCl). For example, the buffer may contain about 2.0 mM $MgCl_2$ and 150 mM NaCl. In a specific embodiment, the aqueous buffer contains a physiological concentration of salt.

In addition, a composition or formulation of the invention may contain additional components in addition to HSA to further stabilize the recombinant adenovirus. Examples of such components include, but are not limited to, carbohydrates and sugars, such as dextrose, sucrose, glucose, and the like, *e.g.*, at a 5% concentration; medium to long chain polyols, such as glycerol, polyethylene glycol, and the like, *e.g.*, at 10% concentration; other proteins; amino acids; nucleic acids; chelators; proteolysis inhibitors;

preservatives; and other components. Preferably, any constituent of a composition of the invention is pharmaceutically acceptable.

The compositions of the invention are particularly suitable for formulation of a recombinant adenovirus for gene therapy. Thus, in a preferred embodiment, the recombinant adenovirus expresses a heterologous protein. Examples of heterologous proteins include, but are by no means limited to, tumor suppressor proteins such as p53; suicide genes such as herpes simplex virus thymidine kinase (HSV-tk); growth factors such as acidic fibroblast growth factor (FGF); angiogenic factors such as FGF or vascular endothelial growth factor (VEGF); trophic factors such at nerve growth factor (NGF), neurotrophic factor-3 (NT-3), NT-4, glial-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF); etc. A more complete list of heterologous proteins for expression in a vector formulated in accordance with the present invention can be found *infra*. In a specific embodiment, the heterologous protein is p53. In another specific embodiment, the heterologous protein is HSV-TK.

Naturally, the present invention further provides a method for preparing a recombinant adenovirus vector formulation comprising preparing an admixture of a recombinant adenovirus and a concentration of HSA effective to stabilize the adenovirus vector at a temperature above the freezing point of water or to enhance a titer of the adenovirus vector compared to a titer in the absence of HSA, or both, in an aqueous buffer. In one embodiment, the temperature is greater than or equal to 4°C and less than 37°C. In a further embodiment, the temperature is greater than or equal to 20°C. Preferably, when the temperature is greater than 4°C, and particularly when the temperature is greater than 20°C, the concentration of HSA is 5%, the pH of the admixture is greater than 8.0, or both.

In a further aspect, the present invention provides a method for stabilizing an adenovirus vector at about 20°C by preparing an admixture of the adenovirus vector in an aqueous composition of Dulbecco's phosphate buffered saline, from about 5% to 15% glycerol, from about 0.25 to 2.0 mM $CaCl_2$, and from about 0.1 to 1.0 mM $MgCl_2$. In a specific embodiment, the concentration of glycerol is 10%, the concentration of $CaCl_2$ is about 1.0 mM, and the concentration of $MgCl_2$ is about 0.5 mM.

The present invention will be further explained in the Drawings and Detailed Description.

DESCRIPTION OF THE DRAWINGS

Figure 1. Plaque Assay - +4°C Storage of 4 Formulations.

Figure 2. Plaque Assay - +20°C Storage of 4 Formulations.

Figure 3. Bioactivity Assay - +4°C Bioactivity for 4 Formulations.

Figure 4. Bioactivity Assay - +20°C Bioactivity for 4 Formulations.

Figure 5. Effect of HSA and Sucrose on Viral Titers at -20°C.

Figure 6. Effect of Buffer, Salt and/or Cations on Viral Titers at +2-10°C.

Figure 7. Effect of HSA on Viral Titer at +2-10°C.

Figure 8. Activity of AdCMVp53 Stored in Formulation 4 at Various pH/1 week.

Figure 9. Comparison of HSA and BSA in viral formulations.

Figure 10. Stability of virus at 37°C for 1 week in different formulations.

Figure 11. Stability of AV1.0HSVTK Stored in Formulation 19 at -70°C, -20°C, +4°C, and +20°C for 1.5, 3.5, and 8.5 months.

DETAILED DESCRIPTION OF THE INVENTION

The present invention advantageously provides a new type of medium which makes it possible to preserve virus vectors, particularly adenovirus vectors, using a formulation that optimally enhances the recombinant virus vector titer, or stabilizes the vector at refrigerator or room temperature, or both.

In specific examples, *infra*, various formulations were tested for their ability to stabilize adenovirus vector preparations for up to 8.5 months, relative to a control stored at -70°C. It was found that a formulation comprising 10 mM Tris-HCl, pH 8.2, 5% HSA, 5% sucrose, 2.0 mM $MgCl_2$, 150 mM NaCl was very stable, and preserved – or even enhanced – vector infectivity for up to 6 months at relatively high temperatures (4°C and 20°C). Additional experiments established that an optimum pH for adenovirus vector stability was above pH 8.0. It was further found that the presence of HSA in the solution increased the viral titer (as measured in a plaque assay). In addition, a second formulation comprising 10mM Tris-HCL, pH 8.4, 5% HSA, 5% sucrose, 2.0mM $MgCl_2$, 150mM NaCl was tested for its ability to stabilize adenovirus vector preparations for at least 8.5 months, relative to a control stored at -70°C. It was found that this 10mM Tris-HCl, pH 8.4, 5% HSA, 5% sucrose, 2.0mM $MgCl_2$, 150mM NaCl formulation was also very stable, preserving viral particle integrity and infectivity (titer) for at least 8.5 months at +4°C and preserving viral particle integrity for at least 8.5 months at +20°C. Thus, the critical variables for a formulation to preserve adenovirus vectors were HSA and pH; sucrose was also found to enhance stability.

The advantage of such a formulation stems from the fact that the solution is available for administration immediately after removal from the storage temperature, without any further manipulation being necessary. It then becomes possible to carry out the removal from storage conditions directly in the clinic, thereby reducing the time between storage and use, which also makes it possible to remain constantly in a sterile formulation and therefore to reduce to a minimum the risks of external contamination. This formulation lacks any toxic agent and may be administered directly to an organism. It may be used for preserving various virus preparations such as viral particles and viral vectors.

As noted above, the invention provides a formulation for the preservation and/or storage of viruses comprising a concentration of HSA effective to stabilize an adenovirus vector at a temperature

above the freezing point of water or to enhance a titer of the adenovirus vector compared to a titer in the absence of HSA, or both, in an aqueous buffer. Preferably, the invention provides a composition comprising a recombinant adenovirus vector and a concentration of HSA effective to stabilize the adenovirus vector at a temperature above the freezing point of water or to enhance a titer of the adenovirus vector compared to a titer in the absence of HSA, or both, in an aqueous buffer. Further, as noted above, the pH of the composition is greater than or equal to 5.0 and less than or equal to 9.0, and preferably, the pH is greater than 7.5. In a specific embodiment, the pH is 8.2 and the concentration of HSA is 5%. In a second specific embodiment, the pH is 8.4 and the concentration of HSA is 5%.

The various aspects of the invention will be set forth in greater detail in the following sections, directed to suitable media and formulations for preserving viral particles and viral vectors. This organization into various sections is intended to facilitate understanding of the invention, and is in no way intended to be limiting thereof.

Definitions

The following defined terms are used throughout the present specification, and should be helpful in understanding the scope and practice of the present invention.

In a specific embodiment, the term "about" or "approximately" means within 20%, preferably within 10%, and more preferably within 5% of a given value or range.

The term "corresponding to" is used herein to refer similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. A nucleic acid or amino acid sequence alignment may include spaces. Thus, the term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

A "formulation" refers to an aqueous or solution medium for the preservation of viral particles and viral vectors, which is directly injectable into an organism. It relates more particularly to a formulation for a recombinant adenovirus vector that optimally enhances the vector titer, or stabilizes the vector at refrigerator or room temperature, or both. It also relates to compositions comprising a recombinant adenovirus vector and a concentration of HSA effective to stabilize the adenovirus vector at a temperature above the freezing point of water or to enhance a titer of the adenovirus vector compared to a titer in the absence of HSA, or both, in an aqueous buffer. The aqueous buffer will include salts or sugars, or both, at about an isotonic concentration.

A "gene" refers to an assembly of nucleotides that encode a polypeptide, and includes cDNA and genomic DNA nucleic acids.

"Human serum albumin" or "HSA" refers to a non-glycosylated monomeric protein of 585

amino acids, with a molecular weight of 66 kD. Its globular structure is maintained by 17 disulphide bridges, which create a sequential series of 9 double loops (Brown, J.R., 1977). "Albumin structure, function and uses", Rosenoer, V.M. *et al.* (eds.), Pergamon Press, Oxford, pp. 27-51). The genes encoding HSA are known to be highly polymorphic, and more than 30 apparently different genetic variants have been identified by electrophoretic analysis under varied conditions (Weitkamp, L.R. *et al.*, 1973. Ann. Hum. Genet., 37:219-226). The HSA gene comprises 15 exons and 14 introns comprising 16,961 nucleotides, from the supposed "capping" site up to the first site of addition of poly(A).

The phrase "pharmaceutically acceptable" refers to molecular entities, at particular concentrations, and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, fever, dizziness and the like, when administered to a human or non-human animal. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in humans or non-human animals.

A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation, *i.e.*, genetic engineering.

A "subject" is a human or a non-human animal who may receive a vector formulated in a composition of the invention.

A "vector" is any means for the transfer of a nucleic acid into a host cell. A vector may be a replicon to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (e.g., plasmid, phage, cosmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, *i.e.*, capable of replication under its own control. The term "vector" as used herein specifically refers to viral means for introducing the nucleic acid into a cell *in vitro*, *ex vivo* or *in vivo*. Viral vectors include retrovirus, adeno-associated virus, pox, baculovirus, vaccinia, herpes simplex, Epstein-Barr and adenovirus vectors, as set forth in greater detail below. The nucleic acid contains a coding region for a gene of interest. In an expression vector, the coding region is operably associated with an expression control sequences, *e.g.*, a promoter. A vector may also contain one or more regulatory regions, and/or selectable markers useful in selecting, measuring, and monitoring nucleic acid transfer results (transfer to which tissues, duration of expression, etc.).

Human Serum Albumin

The HSA used within the framework of the present invention may be either of natural origin (purified HSA) or of the recombinant origin (rHSA). Although the present invention is described primarily in the context of human serum albumin, it has been found *infra* that serum albumin from other

species is equally effective. Thus, the term HSA should be considered to encompass any serum albumin, such as bovine serum albumin (BSA) and murine serum albumin (MSA). Naturally, for delivery of a formulation *in vivo* for gene therapy, it is preferable to use an autologous serum albumin. Thus, for human gene therapy, human serum albumin is desirable and preferred.

A recombinant or natural HSA is advantageously used which meets certain quality criteria (e.g., homogenetic, purity, stability). Thus, the pharmacopoeia sets a number of parameters for the plasma albumin solutions, namely a pH value, a protein content, a polymer and aggregate content, an alkaline phosphatase content and a certain protein composition. It imposes, furthermore, a certain absorbance, the compliance with a test of sterility, with a test for pyrogens and for toxicity (see "Albumini humai solutio", European Pharmacocpoeia (1984), 255). The use of an albumin corresponding to these criteria, although not essential, is particularly preferred.

Advantageously, the compositions according to the invention comprise a purified human plasma albumin or a recombinant human albumin, preferably produced in a eukaryotic host. In addition, the term HSA comprises, for the purpose of the invention, any natural variant of human albumin, resulting from the polymorphism of this protein. It is also possible to use an HSA equivalent, that is to say, any HSA derivative conserving the properties of HSA. These derivatives may be especially amino- (N-) terminal fragments of HSA.

Purification of Natural HSA. Natural HSA is generally produced by purification from biological material of human origin. In particular, it is obtained by conventional techniques for fractionation of plasma obtained from blood donations (Cohn et al., 1946. J. Am. Chem. Soc., 68:459 pp.), or by extraction from the human placenta, according to the technique described by J. Liautraud *et al.* (1973, 13th International IABS Conference, Budapest; A: "Purification of proteins. Development of biological standard", Karger (ed.), Bale, 27:107 pp). Preferably, the purified albumin used within the framework of the present invention is a plasma albumin. Most particularly, a commercial plasma albumin may be used.

Recombinant Production of HSA. The development of genetic engineering and of new extraction and purification techniques has opened the possibility of obtaining, at a lower cost price, improved products of higher purity, of greater stability and without risk of contamination (for example, hepatitis B, hepatitis C, HIV, or infectious prions). Given the importance of the HSA market, the possibility of producing this protein by a recombinant route has been widely studied. Thus, numerous expression systems have been studied for the preparation of the recombinant HSA.

HSA Fermentation. More particularly, as regards the bacterial hosts, genetic engineering can be accomplished in a bacterium, for example, *Escherichia coli,* as a host organism. European patents EP 236 210, EP 200 590, or EP 198 745 describe processes for the production of HSA in *E. coli* using

different expression vectors, different transcriptional promoters, and different secretory signals. Subsequently, secretion of HSA in *Bacillus subtilis* was also carried out (Saunders *et al.*, 1987. J. Bacteriol., 169:2917). As regards the eukaryotic hosts, processes for the production of HSA have been developed using yeasts as a host organism. Thus, it has been possible to demonstrate the production of HSA under the control of the chelatin promoter in *Saccharomyces cerevisiae* (Etcheverry *et al.*, 1986. Bio/Technology, 4:726). The production of HSA has also been mentioned in the brewery yeast during the manufacture of beer, using a post-fermentative process (EP 201 239). Most recently, patent application EP 361 991 describes a particularly efficient system using the yeast *Kluyveromyces* as a host organism, transformed with vectors derived from the plasmid pKD1. Particularly high levels of HSA secreted into the culture medium were able to be obtained with this system. Finally, the production of recombinant HSA has also been described in *Pichia pastoris*, (EP 344 450). In addition, the purification of HSA has also been described (EP 319 067).

Various patents and scientific publications describe methods for expressing a heterologous gene, particularly HSA, in a transgenic animal, optimally in the mammary gland of a ruminant mammal. Such technology is useful for producing a heterologous protein in the milk of the mammal. Examples of production of human serum albumin in transgenic animals include US Patent No. 5,780,009, issued July 14, 1998 to Karatzas *et al.*, directed to direct gene transfer into the ruminant mammary gland. US Patent No. 4,873,316, issued October 10, 1989 to Meade *et al.*, directed to isolation of exogenous recombinant proteins from the milk of transgenic mammals, provides an expression system comprising the mammal's casein promoter, which when transgenically incorporated into a mammal permits the female of that mammal species to produce the desired recombinant protein in or along with its milk. A preferred construct for transgenic expression of HSA is described in US Patent No. 5,648,243, issued July 15, 1997 to Hurwitz *et al.*, directed to a human serum albumin expression construct, the disclosure of which is incorporated herein by reference in its entirety. As disclosed in this patent, efficient expression of HSA is achieved when the human serum albumin sequence comprises at least one, but not all, of the introns in the naturally occurring gene encoding for the HSA protein; preferably the DNA constructs comprise a 5' regulatory sequence which directs the expression and secretion of HSA protein in the milk of a transgenic animal. These patents refer to additional references from the scientific and patent literature for transgenic expression, particularly of HSA. Each of them is incorporated herein by reference it its entirety.

In a specific embodiment of the invention, the recombinant HSA of the invention is Recombumin™ (Centeon). Recombumin™ is a yeast-derived recombinant human albumin, which is structurally identical to plasma-derived human albumin. Recombumin™ is produced using a hybrid secretion leader sequence conjugated to a cDNA which encodes human serum albumin. The cDNA

sequence is disclosed in EP 0 073 646 and the leader sequence is disclosed in US Patent No. 5,302,697. The leader sequence is cloned into an expression cassette in a disintegration vector which is described in US Patent No. 5,637,504. The host yeast cell used to make Recombumin™ includes various mutations, such as gene disruptions of the yeast aspartyl protease gene (WO 95/23857) and gene disruptions of the heat shock protein 150 gene (US 5,783,423).

HSA Formulations

The formulations according to the invention may be prepared in various ways. The different components may be mixed together, and then the viral particle or vector is added to the mixture. It is also possible to mix one or several of the components with the viral particle or vector and then to add the remaining component(s). Preferably, a formulation comprising all of the components is prepared, to which the viral particle or vector is then added. The preparation of the formulation and the addition of the viral particles or viral vectors are performed under sterile conditions.

The respective proportions of the components of the media according to the invention may be adapted by persons skilled in the art according to the viral particle or viral vector considered. As illustrated in the Examples, although certain concentration ranges are preferred, the proportions may be modified.

The formulation according to the invention was discovered in connection with preservation of adenovirus vectors. However, this formulation may very well be useful for preserving other viral vectors.

Viral Particles and Vectors

The viral particles and viral vectors more particularly relevant to the present invention are those which may have be used in gene therapy. A large number of viruses may have their genome modified, on the one hand, so that they lose their ability to multiply while retaining their infectivity, and on the other hand, so as to insert into their genome a nucleic acid sequence of therapeutic interest which will be expressed in the infected cells. Among these viruses, there may be mentioned more particularly, the adenoviruses, the adeno-associated viruses (AAVs), the retroviruses, the herpes viruses and the like.

The present formulation for relatively high temperature storage of a viral particle or vector was specifically developed for storage of an adenovirus vector. However, the invention contemplates that the HSA formulation, particularly with the preferred pH range, may also stabilize or enhance infectivity, or both, of other vectors.

Adenovirus vectors. In a preferred embodiment, the vector is an adenovirus vector. Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a nucleic acid of the invention to a variety of cell types. Various serotypes of adenovirus exist. Of these serotypes, preference is given, within the scope of the present invention, to using type 2 or type 5 human

adenoviruses (Ad 2 or Ad 5) or adenoviruses of animal origin (see WO94/26914). Those adenoviruses of animal origin which can be used within the scope of the present invention include adenoviruses of canine, bovine, murine (example: Mav1, Beard *et al.*, Virology 75 (1990) 81), ovine, porcine, avian, and simian (example: SAV) origin. Preferably, the adenovirus of animal origin is a canine adenovirus, more preferably a CAV2 adenovirus (e.g., Manhattan or A26/61 strain (ATCC VR-800), for example).

Preferably, the replication defective adenoviral vectors of the invention comprise the ITRs, an encapsidation sequence and the nucleic acid of interest. Still more preferably, at least the E1 region of the adenoviral vector is non-functional. The deletion in the E1 region preferably extends from nucleotides 455 to 3329 in the sequence of the Ad5 adenovirus (PvuII-BglII fragment) or 382 to 3446 (HinfII-Sau3A fragment). Other regions may also be modified, in particular the E3 region (WO95/02697), the E2 region (WO94/28938), the E4 region (WO94/28152, WO94/12649 and WO95/02697), or in any of the late genes L1-L5.

In a preferred embodiment, the adenoviral vector has a deletion in the E1 region (Ad 1.0). Examples of E1-deleted adenoviruses are disclosed in EP 185 573, the contents of which are incorporated herein by reference. In another preferred embodiment, the adenoviral vector has a deletion in the E1 and E4 regions (Ad 3.0). Examples of E1/E4-deleted adenoviruses are disclosed in WO95/02697 and WO96/22378, the contents of which are incorporated herein by reference. In still another preferred embodiment, the adenoviral vector has a deletion in the E1 region into which the E4 region and the nucleic acid sequence are inserted (see FR94 13355, the contents of which are incorporated herein by reference).

The replication defective recombinant adenoviruses according to the invention can be prepared by any technique known to the person skilled in the art (Levrero *et al.*, Gene 101 (1991) 195, EP 185 573; Graham, EMBO J. 3 (1984) 2917). In particular, they can be prepared by homologous recombination between an adenovirus and a plasmid which carries, inter alia, the DNA sequence of interest. The homologous recombination is effected following cotransfection of the said adenovirus and plasmid into an appropriate cell line. The cell line which is employed should preferably (i) be transformable by the elements, and (ii) contain the sequences which are able to complement the part of the genome of the replication defective adenovirus, preferably in integrated form in order to avoid the risks of recombination. Examples of cell lines which may be used are the human embryonic kidney cell line 293 (Graham *et al.*, J. Gen. Virol. 36 (1977) 59) which contains the left-hand portion of the genome of an Ad5 adenovirus (12%) integrated into its genome, and cell lines which are able to complement the E1 and E4 functions, as described in applications WO94/26914 and WO95/02697. Recombinant adenoviruses are recovered and purified using standard molecular biological techniques, which are well known to one of ordinary skill in the art.

Other Vectors

Other Viral Vectors. The adeno-associated viruses (AAV) are DNA viruses of relatively small size which can integrate, in a stable and site-specific manner, into the genome of the cells which they infect. They are able to infect a wide spectrum of cells without inducing any effects on cellular growth, morphology or differentiation, and they do not appear to be involved in human pathologies. The AAV genome has been cloned, sequenced and characterized. It encompasses approximately 4700 bases and contains an inverted terminal repeat (ITR) region of approximately 145 bases at each end, which serves as an origin of replication for the virus. The remainder of the genome is divided into two essential regions which carry the encapsidation functions: the left-hand part of the genome, which contains the rep gene involved in viral replication and expression of the viral genes; and the right-hand part of the genome, which contains the cap gene encoding the capsid proteins of the virus.

The use of vectors derived from the AAVs for transferring genes *in vitro* and *in vivo* has been described (see WO 91/18088; WO 93/09239; US 4,797,368, US 5,139,941, EP 488 528). These publications describe various AAV-derived constructs in which the rep and/or cap genes are deleted and replaced by a gene of interest, and the use of these constructs for transferring the said gene of interest in vitro (into cultured cells) or in vivo, (directly into an organism). The replication defective recombinant AAVs according to the invention can be prepared by cotransfecting a plasmid containing the nucleic acid sequence of interest flanked by two AAV inverted terminal repeat (ITR) regions, and a plasmid carrying the AAV encapsidation genes (rep and cap genes), into a cell line which is infected with a human helper virus (for example an adenovirus). The AAV recombinants which are produced are then purified by standard techniques.

In another embodiment the gene can be introduced in a retroviral vector, *e.g.*, as described in Anderson *et al.*, U.S. Patent No. 5,399,346; Mann *et al.*, 1983, Cell 33:153; Temin *et al.*, U.S. Patent No. 4,650,764; Temin *et al.*, U.S. Patent No. 4,980,289; Markowitz *et al.*, 1988, J. Virol. 62:1120; Temin *et al.*, U.S. Patent No. 5,124,263; EP 453242, EP178220; Bernstein *et al.* Genet. Eng. 7 (1985) 235; McCormick, BioTechnology 3 (1985) 689; International Patent Publication No. WO 95/07358, published March 16, 1995, by Dougherty *et al.*; and Kuo *et al.*, 1993, Blood 82:845. The retroviruses are integrating viruses which infect dividing cells. The retrovirus genome includes two LTRs, an encapsidation sequence and three coding regions (gag, pol and env). In recombinant retroviral vectors, the *gag*, *pol* and *env* genes are generally deleted, in whole or in part, and replaced with a heterologous nucleic acid sequence of interest. These vectors can be constructed from different types of retrovirus, such as, HIV, MoMuLV ("murine Moloney leukaemia virus" MSV ("murine Moloney sarcoma virus"), HaSV ("Harvey sarcoma virus"); SNV ("spleen necrosis virus"); RSV ("Rous sarcoma virus") and Friend virus. Defective retroviral vectors are disclosed in WO95/02697.

Regulatory Regions. Expression of a polypeptide from a vector of the invention may be controlled by any regulatory region, *i.e.*, promoter/enhancer element known in the art, but these regulatory elements must be functional in the host tissue, such as a target tumor, selected for expression.

The regulatory regions may comprise a promoter region for functional transcription in the host cell, as well as a region situated 3' of the gene of interest, and which specifies a signal for termination of transcription and a polyadenylation site. All these elements constitute an expression cassette.

Promoters that may be used in the present invention include both constitutive promoters and regulated (inducible) promoters. The promoter may be naturally responsible for the expression of the nucleic acid. It may also be from a heterologous source. In particular, it may be promoter sequences of eukaryotic or viral genes. For example, it may be promoter sequences derived from the genome of the cell which it is desired to infect. Likewise, it may be promoter sequences derived from the genome of a virus, including the adenovirus used. In this regard, there may be mentioned, for example, the promoters of the E1A, MLP, CMV and RSV genes and the like.

In addition, the promoter may be modified by addition of activating or regulatory sequences or sequences allowing a tissue-specific or predominant expression (enolase and GFAP promoters and the like). Moreover, when the nucleic acid does not contain promoter sequences, it may be inserted, such as into the virus genome downstream of such a sequence.

Some promoters useful for practice of this invention are ubiquitous promoters (*e.g.*, HPRT, vimentin, actin, tubulin), intermediate filament promoters (*e.g.*, desmin, neurofilaments, keratin, GFAP), therapeutic gene promoters (*e.g.*, MDR type, CFTR, factor VIII), tissue-specific promoters (*e.g.*, actin promoter in smooth muscle cells), promoters which are preferentially activated in dividing cells, promoters which respond to a stimulus (*e.g.*, steroid hormone receptor, retinoic acid receptor), tetracycline-regulated transcriptional modulators, cytomegalovirus immediate-early, retroviral LTR, metallothionein, SV-40, E1a, and MLP promoters. Tetracycline-regulated transcriptional modulators and CMV promoters are described in WO 96/01313, US 5,168,062 and 5,385,839, the contents of which are incorporated herein by reference.

Thus, the promoters which may be used to control gene expression include, but are not limited to, the cytomegalovirus (CMV) promoter, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster *et al.*, 1982, Nature 296:39-42); prokaryotic expression vectors such as the b-lactamase promoter (Villa-Kamaroff, *et al.*, 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *tac* promoter (DeBoer, *et al.*, 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from

recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38:639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38:647-658; Adames *et al.*, 1985, Nature 318:533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert *et al.*, 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5:1639-1648; Hammer *et al.*, 1987, Science 235:53-58), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey *et al.*, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram *et al.*, 1985, Nature 315:338-340; Kollias *et al.*, 1986, Cell 46:89-94), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48:703-712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, Science 234:1372-1378).

Therapeutic Genes. Examples of heterologous proteins expressed by vectors include, but are by no means limited to, tumor suppressor proteins such as p53; suicide genes such as herpes simplex virus thymidine kinase (HSV-tk); growth factors such as acidic fibroblast growth factor (FGF); angiogenic factors such as FGF or vascular endothelial growth factor (VEGF); trophic factors such at nerve growth factor (NGF), neurotrophic factor-3 (NT-3), NT-4, glial-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF); etc. In a specific embodiment, the heterologous protein is p53. In a second specific embodiment, the heterologous protein is HSV-TK.

Naturally, the present invention further provides a method for preparing a recombinant adenovirus formulation comprising preparing an admixture of a recombinant adenovirus and a concentration of HSA effective to stabilize the adenovirus vector at a temperature above the freezing point of water or enhance a titer of the adenovirus vector compared to a titer in the absence of HSA, or both, in an aqueous buffer. In one embodiment, the temperature is greater than or equal to 4°C and less than 37°C. In a further embodiment, the temperature is greater than or equal to 20°C. Preferably, when the temperature is greater than 4°C, and particularly when the temperature is greater than 20°C, the

concentration of HSA is 5%, the pH of the admixture is greater than 8.0, or both.

In a further aspect, the present invention provides a method for stabilizing an adenovirus vector at about 20°C by preparing an admixture of the adenovirus vector in an aqueous composition of Dulbecco's phosphate buffered saline, from about 5% to 15% glycerol, from about 0.25 to 2.0 mM $CaCl_2$, and from about 0.1 to 1.0 mM $MgCl_2$. In a specific embodiment, the concentration of glycerol is 10%, the concentration of $CaCl_2$ is about 1.0 mM, and the concentration of $MgCl_2$ is about 0.5 mM.

The use of a formulation according to the invention makes it possible to preserve viral particles and viral vectors and to administer it directly into a subject, without a centrifugation or washing stage, with a good viability and/or without affecting its capacity to infect a susceptible cell of the organism.

To this end, the present invention also relates to preparations containing the preservation formulation according to the invention and viral particles or viral vectors, as well as to a process for the storage of the viral particles or viral vectors. The viral particles or viral vectors may be packaged directly into the formulation according to the invention. As regards viruses, these are previously purified as described herein (e.g., by centrifugation on a cesium chloride gradient, column chromatography, plaque purification, and the like). They may be packaged at the rate of 10^4 to 10^{15} particles per ml, preferably 10^5 to 10^{10}, or more preferably, 10^9-10^{13}. The viral particles or viral vectors may then be packaged in the formulation according to the invention, in an appropriate container. It may an ampoule, a tube, especially a cryotube, a bag, a vial, a flask, and the like. The container is previously sterilized and the packaging operations are performed under sterile conditions.

A medium or formulation according to the invention allows the storage and preservation of viral particles or viral vectors under conditions that preserve good viability. The formulation according to the invention may, in particular, allow the storage of the particles or vectors at a temperature above the freezing point of water. In a preferred embodiment, the media according to the invention may allow the storage of recombinant adenovirus vectors at a temperature above the freezing point of water or enhance a titer of the adenovirus vector compared to a titer in the absence of HSA, or both, in an aqueous buffer.

Pharmaceutical Compositions. For their use according to the present invention, the vectors, either in the form of a virus vector or virus particle are preferably combined with one or more pharmaceutically acceptable carriers for an injectable formulation. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. These may be in particular isotonic, sterile, saline solutions

(monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, allow the constitution of injectable solutions.

The preferred sterile injectable preparations can be a solution or suspension in a nontoxic parenterally acceptable solvent or diluent. Examples of pharmaceutically acceptable carriers are saline, buffered saline, isotonic saline (e.g., monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride, or mixtures of such salts), Ringer's solution, dextrose, water, sterile water, glycerol, ethanol, and combinations thereof. 1,3-butanediol and sterile fixed oils are conveniently employed as solvents or suspending media. Any bland fixed oil can be employed including synthetic mono- or di-glycerides. Fatty acids such as oleic acid also find use in the preparation of injectables.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15 percent, preferably by at least 50 percent, more preferably by at least 90 percent, and most preferably prevent, a clinically significant deficit in the activity, function and response of the subject. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the subject.

Administration of Compositions. According to the invention, the composition of the invention may be introduced parenterally or transmucosally, *e.g.*, orally, nasally, or rectally, or transdermally. Preferably, administration is parenteral, *e.g.*, via intravenous injection, and also including, but is not limited to, intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial administration. For gene therapy of a cancer, the administration of the composition may also be introduced by injection into a tumor or into tissues surrounding the tumor.

The preferred route of administration to a tumor is by direct injection into the tumor. The tumor can be imaged using any of the techniques available in the art, such as magnetic resonance imaging or computer-assisted tomography, and the therapeutic composition administered by stereotactic injection, for example. Alternatively, if a tumor target is characterized by a particular antigen, a vector of the invention can be targeted to the antigen as described above, and administered systemically or subsystemically, as appropriate, *e.g.*, intravenously, intraarterioally, intraperitoneally, intraventricularly, etc.

The virus doses used for the administration may be adapted as a function of various parameters, and in particular as a function of the site of administration considered, the number of injections, the gene to be expressed or alternatively the desired duration of treatment. In general, the recombinant adenoviruses according to the invention are formulated and administered in the form of doses of between 10^4 and 10^{14} pfu, and preferably 10^5 to 10^{11} pfu. The term pfu (plaque forming unit) corresponds to the infectivity of a virus solution, and is determined by infecting an appropriate cell culture and measuring,

generally within 15 days, the number of plaques of infected cells. For p53 adenovirus and HSV-TK adenovirus, typically the number of plaques are counted at day 7. For other viruses, evaluation is made at day 12-14. The technique for determining the pfu titre of a viral solution are well documented in the literature.

Thus, the compositions of the invention can be delivered by intravenous, intraarterial, intraperitoneal, intramuscular, pulmonary, or subcutaneous routes of administration. Alternatively, the compositions, properly formulated, can be administered by nasal or oral administration. A constant supply of the viral particles or viral vectors can be ensured by providing a therapeutically effective dose (*i.e.*, a dose effective to induce metabolic changes in a subject) at the necessary intervals, *e.g.*, daily, weekly, monthly, etc. These parameters will depend on the severity of the disease condition being treated, other actions, such as diet modification, that are implemented, the weight, age, and sex of the subject, and other criteria, which can be readily determined according to standard good medical practice by those of skill in the art.

A subject in whom administration of a viral particle or viral vector within the scope of the invention is administered is preferably a human, but can be any animal. Thus, as can be readily appreciated by one of ordinary skill in the art, the methods and pharmaceutical compositions of the present invention are particularly suited to administration to any animal, particularly a mammal, and including, but by no means limited to, domestic animals, such as feline or canine subjects, farm animals, such as but not limited to bovine, equine, caprine, ovine, and porcine subjects, wild animals (whether in the wild or in a zoological garden), research animals, such as mice, rats, rabbits, goats, sheep, pigs, dogs, cats, etc., avian species, such as chickens, turkeys, songbirds, etc., *i.e.*, for veterinary medical use.

The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention.

EXAMPLES

General Molecular Biology

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL

Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

EXAMPLE 1: Stability Testing of Adenovirus Formulations at Above Freezing Temperatures.

Initially, the adenovirus preparations were stored at -70°C in buffered glycerol solutions to provide the best long term stability. Since -70°C storage may not be available in the clinical setting, a formulation which will preserve the virus at -20°C or higher temperatures is needed.

This Example summarizes six month data for four Formulations tested at five storage temperatures. This study was designed to determine optimal formulation of an adenovirus vector to achieve at least one (1) year stability when stored frozen at -20°C, or as a liquid at +4°C or +20°C storage temperatures. A positive control consisted of the virus stored in Dulbecco's phosphate buffered saline (DPBS), 10% glycerol, 1.0mM $CaCl_2$ and 0.5mM $MgCl_2$ at -70°C. Test samples for all four Formulations were passed though Sephadex G25 column for buffer exchange. Triplicate virus samples and positive controls were tested at one month intervals for 3 consecutive months, then every one and one-half months thereafter. Viral titer in plaque forming units (pfu) was determined by plaque assay using 293 cells. An analytical HPLC method was used to determine viral particle/ml measurements by peak area of an optical density (OD) at 260nm. Viral particles were determined by UV measurements made on Formulations not containing HSA. The Bioactivity assay was used as an additional test for viral activity at selected time points.

Methods

Virus Sample Preparation: A type 5 adenovirus comprising the p53 gene under the control of the cytomegalovirus promoter, Ad5CMVp53 (as described in International Patent Application No. US95/04898), in DPBS + 10% glycerol, 0.2 μm filtered, in 10 ml vials was used in these experiments. The virus titer was estimated at $1.38x10^{11}$ pfu/ml and $3.44x10^{12}$ particles/ml. On the first day of the study, thirteen vials of Ad5CMVp53 were thawed at room temperature and pooled. OD measurements were taken at 260nm on the pooled material to determine the original particles/ml. Thirty ml of the pool were then run through a Sephadex G25 column (200ml; Pharmacia Catalog No. 17-0033-01, lot 241586) for buffer exchange into each of the formulations. Buffer exchange was performed to place the virus into appropriate formulations for testing. Four Formulations were tested and are defined in Table 1. Only 0.5% HSA was present in Formulations 2, 3 and 4 at the time of

Table 1: Test Formulations.

Form 1: DPBS, 10% glycerol, 1.0mM $CaCl_2$ and 0.5mM $MgCl_2$
Form 2: DPBS, 5% HSA, 5% Sucrose, 1.0mM $CaCl_2$ and 0.5mM $MgCl_2$
Form 3: 10mM Tris-HCL, pH 7.5, 5% HSA, 5% Sucrose, 2.0mM $MgCl_2$, 150mM NaCl
Form 4: 10mM Tris-HCL, pH 8.2, 5% HSA, 5% Sucrose, 2.0mM $MgCl_2$,150mM NaCl

buffer exchange. Post-buffer exchange OD measurements were taken at 260nm to determine the particle dilution that occurred during the exchange process. Particle dilution was reported as percent yield. After the buffer exchange and OD measurements were completed, HSA was added to the appropriate Formulations. Dilution of the samples by buffer exchange and addition of HSA was calculated by the following equation: (Column Dilution)X(% yield)X(HSA Dilution). All samples were diluted to a final dilution of 3.2 fold from the original pool concentration. After the final dilution, all samples were stored overnight at +4°C and were aliquoted and tested the next day. All four virus Formulation samples were aliquoted into labeled glass amber vials (Kimble #203), 0.42 ml each and stored at the appropriate temperatures, -20°C (VWR Scientific freezer), -4°C (LabLine Ambi HiLo Chamber), +4°C (VWR Scientific 2-door deli case Model GDM-49, +20°C, and +37°C (Queue Systems, Inc., Cell Star Incubator Model No. QWJ8000ABA). The samples for -20°C storage were frozen to -40°C in a controlled rate freezer prior to storage at -20°C. The temperatures of -4°C, +20 and +37 were used as elevated temperatures with which to determine the "accelerated" stability of the virus formulations at -20°C.

Positive Control Sample Preparation: The remaining volume of original virus pool, after test samples had been removed for buffer exchange, was diluted 3.2 fold to match the dilution of the test samples described above. The control was diluted in DPBS, 10% glycerol, 1.0mM $CaCl_2$ and 0.5mM $MgCl_2$ to mimic the current Ad5CMVp53 formulation. Control virus was aliquoted, 0.42 ml per cryovial (Nalgene No. 5000-0020, lot 072381) and stored at -70°C (BioFreezer, Forma Scientific Model No. 8328).

Formulation Analysis: All four Formulations were analyzed after 0, 1, 2, 3, 4.5, and 6 months of storage at different temperatures with respect to several parameters as described in Table 2. However, all temperatures were not tested at each time point due to previous results which indicated that further analysis was not required.

Plaque Assay Analysis: Viral titer was determined by the plaque assay on 293 cells, human embryonic kidney cells transformed with sheared human Ad5 DNA (complement E1 region). At designated time points, triplicate test samples and triplicate positive controls were tested for plaque forming units. Two days prior to testing, 293 cells (ATCC® Catalog No. CRL 1573) were seeded onto 6 cm tissue culture dishes. On the day of testing, the virus samples and positive control were serially diluted in MEM+0.5% HEPES. Two or more dilutions which were expected to yield countable plaques were used to infect 293 cells. Three confluent 6 cm dishes of 293 cells were infected with 0.5 ml of each virus sample and positive control dilution. The dishes were incubated at 37°C, 5.0% CO_2 and 95% relative humidity for 2 hours with rocking every 15 minutes. After incubation, the samples were aspirated off and the dishes were overlaid with a 0.5% SeaKem Agarose,

MEM, 7.5% FBS overlay. The dishes were then incubated at 37°C, 5.0% CO_2 and 95%

Table 2: Summary of 6 Month Formulation Analysis.

Month	Plaque Assay	HPLC	OD by UV	Bioactivity Assay
0	Yes	Yes	Yes	No
1	Yes	Yes	Yes	No
2	Yes	Yes	Yes	Yes
3	Yes	Yes	Yes	Yes
4.5	Yes	Yes	Yes	No
6	Yes	Yes	Yes	Yes

relative humidity. After 5 days of incubation, the plates were stained with 0.1% neutral red in MEM overnight at 37°C. The next day, the stain was aspirated off and the plaques were counted. Each dilution's titer was determined in plaque forming units per ml (pfu/ml) by the following equation: [(Average Count X Dilution)/ Volume]. A sample's pfu/ml titer was determined by averaging the pfu/ml of the dilutions. Triplicate sample averages were determined for each time point. Results were plotted in Microsoft EXCEL for further analysis.

HPLC Analysis: HPLC peak area comparisons were performed to determine the virus concentration in terms of viral particles between samples and positive controls. Each triplicate sample and positive control was tested on a Waters analytical HPLC system with Millennium software. Briefly, 100 µl of test sample and positive control were injected into a Resource Q 1ml, 6.4x30 mm column (Pharmacia Catalog No. 17-1177-01), using a flow rate at 1 ml/minute. The samples are stored at 2-8°C in the Waters autosampler during the entire run. The running gradient consisted of line A: Buffer A (50mM Tris, pH 7.5), line B: Buffer B (50mM Tris + 1 M NaCl, pH 7.5), line C: Buffer C (0.5 N NaOH, cleaning solution, used to clean the column after each virus run), and line D: Buffer D (water). The intact adenovirus is eluted between 18-22 minutes after the start of the run. The peak area of every single peak eluted in the entire gradient is reported by the Millenium software. Only the adenovirus peak which is eluted as a single peak at the 18-22 minute range was used as an indication of the virus particle concentration. The peak area for the virus peak was integrated and reported by Millennium software. Data were plotted and analyzed in Microsoft EXCEL.

Optical Density by UV Analysis: Viral particle per ml concentrations were determined for the positive control and Formulation 1 samples which do not contain HSA. HSA containing formulations were not tested for particles by OD because 5% HSA interfered with the reading (data not shown). Briefly, 50µl of 1% SDS and 50µl of sample were incubated for 20 minutes at room temperature, then diluted 1:10 with deionized water. Samples were read at 260 nm and 280 nm. The ratio of 260/280 was calculated to determine the purity. The OD_{260} extinction coefficient of 1 = 10^{12} particles/ml was used to calculate the viral particles/ml of the test sample and positive control. Data were plotted and analyzed in

Microsoft EXCEL.

Bioactivity Assay Analysis: The p53 transgene expression was determined by the inhibition of proliferation of Saos-LM2 cells, a human tumor cell line derived from a lung metastasis from osteogenic sarcoma. In this test, 3 x 10^3 Saos-LM2 cells/well were seeded onto 96 well titer plates in DMEM-high glucose (HG) + 10% FBS-heat inactivated (HI) 3 days prior to infection with the virus samples diluted to various Multiplicities of Infection (10, 20, 40, 80, and 160 MOI) in DMEM-HG + 10% FBS-HI. The infected cells were incubated at 37°C for 4 days and then stained with alamar blue, an indicator of cell growth, and incubated for 8 hours. The plates with alamar blue were then read at 570 nm and 595 nm to determine OD. Optical density was calculated using the following equation: $OD = Absorbance_{570nm} - Absorbance_{595nm}$. The inhibition of proliferation was plotted as a percent reduction of alamar blue in Microsoft EXCEL for further analysis.

Statistical Analysis: Mean and standard deviations were made for triplicate samples.

Results

Plaque Assay Results: The results of the plaque assays for adenovirus stability at 4°C and 20°C are shown in Figures 1 and 2. At -20°C, all of the tested formulations were stable for six months (data not shown). At 4°C and 20°C, Formulation 4 demonstrated increased stability at all time points (Figures 1 and 2). Surprisingly, at -4°C, Formulation 4 demonstrated the same pfu activity as the other formulations (data not shown). At 37°C, all of the formulations were unstable (data not shown).

Storage at +4°C allowed the determination of the stability differences between the Formulations. Formulation 1 performed better than Formulations 2 and 3 following 1 month of storage at +4°C, however it was reduced significantly in pfu/ml by greater than 1 log at month 3 (Figure 1). Formulation 1 did not perform as well as Formulation 4 when stored at +4°C at all tested time points (Figure 1). Formulations 2 and 3 showed an initial decrease in titer following 1 month storage at +4°C (Figure 1). At +4°C, Formulation 4 remained essentially unchanged up to 3 months and showed activity loss starting at 4.5 month (Figure 1).

Storage at +20°C also permitted the determination of the stability differences between formulations. Formulations 1 and 4 showed the best retention of viral activity at +20°C for 6 months (Figure 2). Formulation 4 showed the lowest decrease over 6 months (Table 3) which is only 0.33 logs. Formulation 1 appears to be the second best formulation, as it only decreased by 2 logs. The "too numerous to count" (TNTC) data points in Figure 2 indicated that real titers should be higher than the reported values. Starting at one month, Formulations 2 and 3 showed a reduction in titer when stored at +20°C (Figure 2), which was supported by both the HPLC and Bioactivity assays. Formulations 2 and 3 were less favorable for long term stability than were Formulations 1 and 4.

Table 3: Viral Titer Summary: Plaque Assay Results

Log Delta:	T = 0 -20°C	T = 6M -70°C		T = 6M -20°C		T = 6M +4°C		T = 6M +20°C	
	pfu/ml	pfu/ml	Log =	pfu/ml	Log =	pfu/ml	Log =	pfu/ml	Log =
(+) Control	4.6E+10	3.9E+11	0.93						
Form 1	5.5E+10			3.2E+11	0.76	1.4E+07	-3.59	7.0E+08	-1.90
Form 2	1.5E+12			2.6E+12	0.24	9.4E+04	-7.20	5.9E+05	-6.41
Form 3	1.0E+12			5.6E+12	0.75	2.4E+07	-4.62	2.3E+06	-5.64
Form 4	1.6E+12			4.5E+12	0.45	1.2E+09	-3.12	7.5E+11	-0.33

Note 1: Log = is calculated by (Log T=6 pfu/ml) - (Log T=0 pfu/ml). A (-) result = loss, a (+) result = gain.

Note 2: The samples stored at -4C and +37C samples lost more than 4 logs of activities at T= 1 month and were discontinued in the testing scheme.

Storage at -4°C and at +37°C for one month showed a drastic loss in activity for all four Formulations (data not shown). The decreased activity of the -4°C and +37°C samples were supported by results of HPLC and OD analyses. All samples stored at -4°C were liquid, not frozen. This may have been due to the large amounts of excipients in the formulations preventing complete freezing at -4°C. Therefore, samples stored at -4°C may have been in a transition phase between liquid and solid where ice crystals may have caused damage. In addition, the -4°C samples cannot be used for accelerated shelf life calculations for -20°C frozen storage because they were not frozen. The -4°C and +37°C samples were discontinued at month 1.

In summary, the 6 month viral titer data indicated that Formulation 4 represents a viral formulation that is preferable for preserving viral stability and activity (infectivity), especially at +20°C.

HPLC Results: HPLC peak area provides for the quantitation of viral particles. The HPLC assay variation has been determined to be $\pm$ 3%. Due to the small standard deviations in the HPLC peak areas, any small variation between time points may be statistically significant. The HPLC peak area were plotted by temperature over time for all four Formulations and correlated well with the pfu/ml results described above.

At time 0, all four Formulations have similar viral particles/ml. Although the peak area values showed a statistically significant variation, the difference between Formulations at time point 0 was less than 7%. This low percent variation indicates that a large variation in aliquoting and dilutions did not occur among the four formulations.

At -20°C, all four formulations were relatively unchanged over time (data not shown).

At +4C, all four formulations except Formulation 4 showed significant loss in viral particles

from 1-6 months. Formulation 4 maintained the viral particle concentration until 4.5 month, when its peak area was reduced similar to the level of Formulations 1, 2, and 3 (data not shown). At +20°C, only Formulations 2 and 3 showed consistent loss of viral particles from 1 to 6 months, while Formulations 1 and 4 maintained most of their viral particles (data not shown).

Similar to the plaque assay data, for formulations stored at -4°C and +37°C, all four Formulations showed a drastic decrease in HPLC peak area at 1 month (data not shown).

In summary, the HPLC results showed a similar trend in stability as the plaque assay results for each formulation. All four Formulations showed significant viral titer decreases along with significant viral particle decreases after just one month when stored at -4°C or +37°C.

Bioactivity Assay Results: The Formulations were plotted by temperature over time, at an MOI of 20. Results were reported as percent inhibition of cell growth. Increased cell death by viral infection caused an increase in inhibition of cell growth as determined by a reduction of alamar blue in infected cells compared to uninfected cells.

The bioactivity data also support the stability results obtained from the pfu and HPLC assays. Formulation 4 at +4°C for 6 months retained >50% of cell growth inhibition, which is the clinical specification for this product. At -20°C, all formulations performed similarly (data not shown). Formulation 1 performed poorly at 2 months, but not at 3 and 6 months, indicating that the 2 month data were anomalous (data not shown).

At +4°C, Formulations 1, 2 and 3 did not perform as well with respect to preserving bioactivity as Formulation 4 for months 2 and 3 (Figure 3). The data demonstrated that Formulation 4 retained bioactivity similar to the positive control at both 2 and 3 months, and lost the activity at 6-month. These data correlated well with the plaque assay and HPLC results presented above.

At +20°C, as in the plaque assay results, both Formulations 1 and 4 retained bioactivity similar to the positive control out to month 3, and started to show activity loss at 6-months (Figure 4). Formulation 4 still gave the best bioactivity among all formulations. Formulations 2 and 3 did not perform well following 2 or 3 months storage (Figure 4).

Discussion

The results of this study indicated that all four formulations analyzed did not significantly decrease viral titer (pfu/ml) over a 3 month period when stored at -20°C. The bioactivity data support this conclusion. All four formulations proved stable at -20°C for 6 months.

Formulations 1, 2 and 3 decreased viral titer by more than 2 logs when stored at +4°C and, by more than 53% when measured by HPLC peak area. At +4°C storage, Formulation 4 was the most stable over the 3 month storage period, as evidenced by the plaque assay and HPLC peak area measurements of

particle/ml which were reduced by only 14.8%. These conclusions were supported by the bioactivity assay results. Thus, Formulation 4 proved most stable at +4°C for 3 months.

The viral titer for Formulations 1 and 4 remained stable, within assay variation, for 3 months when stored at +20°C, but the titer of Formulation 1 decreased by 2 logs and the titer of Formulation 4 decreased by 0.3 logs between 3-6 months. Viral particle measurement by HPLC peak area demonstrated a reduction of only 11.1% for Formulation 1 and 23.8% for Formulation 4 by month 3, but 15% for Formulation 1 and 8% for Formulation 4 at 6 months. Virus stored in Formulations 2 and 3 were significantly reduced in viral titer: by more than 2 logs at month 3. The number of viral particles per ml, as measured by HPLC, decreased by more than 30%. The bioactivity decrease correlated with these conclusions. Thus, Formulation 4 proved most stable at +20°C for 6 months.

All four formulations were unstable when stored at -4°C, resulting in a reduction in viral titer that was greater than 4 logs at one month, and greater than 71% loss in particles when measured by HPLC peak area. In addition, storage of all four formulations at 37°C for 1 month resulted in decreased viral titer that was greater than 5 logs, and a loss of more than 85% of particles when measured by HPLC peak area. Thus, none of the formulations analyzed proved stable at -4°C or +37°C.

According to this study, Formulation 4, 10mM Tris buffer at pH 8.2, 5% HSA, 5% sucrose, 2mM $MgCl_2$ and 150 mM NaCl, was the most favorable for long term viral storage. The results from the plaque assay, HPLC particle/ml measurement, and bioactivity assays correlated well with each other and suggested that Formulation 4 retains viral activity for 6 months when stored at -20°C and +20°C. These data also suggest that the original formulation of DPBS, 10% glycerol, 1.0mM $CaCl_2$ and 0.5mM $MgCl_2$ (Formulation 1) retained most of its activity for 3 months at -20°C and +20°C. Over time, Formulation 1, resulted in a decline in both viral titer and particles/ml when stored at +4°C. Formulation 4 started to show the loss of activity and viral particles after 3 months.

EXAMPLE 2: -20°C Screen of Formulations to Preserve Viral Titers.

These experiments screen several formulations for their ability to preserve virus activity as measured by plaque assay when stored at -20°C. These experiments tested various buffers and excipients for their effects on virus titer at -20°C. With regard to the type of buffer, salt concentration, presence of divalent cations, and length of time at -20°C (1 day versus 9 days), there were no differences observed in virus titer by the plaque assay. However, the addition of sucrose and/or human serum albumin (HSA) demonstrated an unexpected increase in the virus infectivity compared to the control.

Methods

Preparation of Virus Sample Formulations: A type 5 adenovirus containing the p53 gene under the control of the cytomegalovirus (CMV) promoter, Ad5CMVp53, was used for these experiments. This material was diluted into various test formulations, frozen, thawed, and analyzed by plaque assay. The stock virus was diluted 100 fold into the various test formulations, which are listed in Table 4. The buffer components were either 10 mM phosphate buffered saline (PBS), pH 7.2, or 10 mM Tris buffered saline, pH 7.5. Additional buffer excipients included 50mM, 150 mM, or 300mM NaCl, divalent cations (1mM $CaCl_2$ and 0.5mM $MgCl_2$), 5% sucrose, and 1%, 5%, or 10% HSA. The samples were aliquoted and frozen in vials using the controlled rate freezer, and transferred to a -20°C freezer for storage, as described in Example 1. At 1 day and 9 days after freezing, the samples were thawed and tested for virus activity by the standard plaque assay.

Preparation of Control Virus Samples: Ad5CMVp53 used in the test formulation preparations above was diluted into the standard buffer (DPBS, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 10% glycerol, pH 7.2.) and treated identically to the test samples as described above.

Plaque Assay: The viral plaque assay was performed as described in Example 1. The duplicate plate counts for each dilution were averaged. The calculations for pfu/ml include the 100 fold dilution into test buffers and were calculated as described in Example 1.

Statistics: The data was transferred to Systat version 5 software for statistical analysis. Linear regression and t-tests were performed to determine differences between groups, using an alpha value of $p=0.05$.

Table 4. Test Formulations.

Form. ID	buffer	[NaCl]	cations	other
1	PBS	50 mM	yes	10% glycerol
2	PBS	50 mM	no	10% glycerol
3	PBS	150 mM	yes	10% glycerol
4	PBS	150 mM	no	10% glycerol
5	PBS	300 mM	yes	10% glycerol
6	PBS	300 mM	no	10% glycerol
7	Tris	50 mM	yes	10% glycerol
8	Tris	50 mM	no	10% glycerol
9	Tris	150 mM	yes	10% glycerol
10	Tris	150 mM	no	10% glycerol
11	Tris	300 mM	yes	10% glycerol
12	Tris	300 mM	no	10% glycerol
13	PBS	150 mM	yes	5% sucrose, 1% HSA
14	PBS	150 mM	yes	1% HSA
15	PBS	150 mM	yes	5% sucrose, 5% HSA
16	PBS	150 mM	yes	5% HSA
17	PBS	150 mM	yes	5% sucrose, 10% HSA
18	PBS	150 mM	yes	10% HSA

Results and Discussion

Effect of Buffer, Salt and Divalent Cations on Viral Titer at -20°C: Formulations 1-12 were analyzed to determine the effects of buffer, salt concentration and/or divalent cation addition on viral titers. The results of the plaque assays after 1 day and 9 days of storage at -20°C for Formulations 1-12 are shown in Table 5. The samples from the frost-free freezer were used for day 1 testing.

It is apparent from the data that there are no great differences to be seen. In fact, the overall confidence of variation (CV = (SD/mean)x100%) for each day obtained by combining all formulations is about the same as that for replicates within an experiment for results of intra-assay variation.

A multiple regression analysis was performed for viral titer (pfu/ml) with respect to buffer,

Table 5. Plaque Assay Results (pfu/ml) at -20°C.

Formulation	Day 1	Day 9
1	4.40×10^9	4.62×10^9
2	4.34×10^9	3.88×10^9
3	2.56×10^9	3.44×10^9
4	3.06×10^9	4.50×10^9
5	4.62×10^9	3.94×10^9
6	3.86×10^9	3.56×10^9
7	3.62×10^9	3.90×10^9
8	4.58×10^9	3.62×10^9
9	5.12×10^9	4.04×10^9
10	3.78×10^9	5.06×10^9
11	4.60×10^9	2.86×10^9
12	3.56×10^9	3.02×10^9
mean	4.01×10^9	3.87×10^9
SD	7.37×10^8	6.39×10^8
CV	18%	17%

salt concentration, presence of divalent cations, and 1 versus 9 days of -20°C exposure, but no significant differences were found (data not shown). With respect to the PBS and Tris buffers, no difference was found ($p = 0.784$). There was no relationship for the effect of salt concentration, from 50 mM to 150 mM ($p=0.322$) and the presence of divalent cations had no effect ($p=0.801$). In addition, the length of storage time at -20°C, 1 day versus 9 days, also showed no difference ($p=0.643$).

Effect of HSA and Sucrose at -20°C: Formulations 13-18 were analyzed to determine the effects of the addition of HSA and sucrose to PBS on viral titer. The results of the day 9 plaque assay are shown in Table 6. The combined effects of HSA and sucrose on viral titer are shown in Figure 5.

There was an increase in the viral titer with the addition of HSA alone and with HSA + sucrose. When the HSA + sucrose Formulation was compared to the control, a significant difference was seen ($p=0.04$). A significant difference was also seen for all samples with HSA as compared to the control ($p=0.004$). The best effects in this single data point experiment were observed with 5% HSA, at either 5% or 0% sucrose. Good results were also found for 5% sucrose, 1% HSA.

Table 6. The Effect of HSA and Sucrose on Viral Titers at -20°C.

Form. ID	sucrose %	HSA %	pfu/ml
13	5	1	6.10×10^9
14	0	1	3.76×10^9
15	5	5	6.66×10^9
16	0	5	6.46×10^9
17	5	10	5.44×10^9
18	0	10	5.92×10^9

EXAMPLE 3: Formulation Screening for +2-10°C storage.

For future clinical studies, it will be preferred to store virus at temperatures of -20°C and above. These experiments are a continuation of those of Example 2, and are designed to screen a number of Formulations for storage at +2-10°C. As described in Example 1 and as determined within this Example, Formulations with HSA preserved all of the virus activity when stored at +2-10°C, while those without HSA lost varying amounts of activity. The experiments described within this Example tested various buffers and excipients for their effects on virus titer when stored at +2-10°C.

Methods

Preparation of Virus Sample Formulations: The Ad5CMVp53 stock virus, dilutions and Formulations described in Example 2 were used in this study. The Formulations are listed in Table 4. The prepared Formulation samples for this study were kept at +2-10°C in a refrigerator. After 14 days, the samples were removed and tested for virus activity by the standard plaque assay. The viral plaque assay was performed and the viral titers were calculated as described above in Example 1.

Statistics: For this experiment, the plaque values from only one dilution (3.3×10^{-8}) were used for analysis, as plaque counts were low, and only this dilution consistently produced plaque numbers in the desired range. The data was transferred to Systat version 5 software for statistical analysis. Linear regression and t-tests were performed to determine differences between groups, using an alpha value of $p=0.05$.

Results and Discussion

Effects of Storage at +2-10°C: The results of the plaque assay after 14 days of storage at +2-10°C are shown in Figure 6. The values in Figure 6 were taken from a single assay dilution for each sample. No error bars are shown as there were no replicates for each condition.

As shown in Figure 6, there were distinct groups of virus activity. The viral titers of Formulations 1-10 were fairly consistent, while Formulations 11 and 12 (which contained Tris buffer with 300 mM NaCl) were considerably lower. Formulations 13-18, which contained HSA, showed increased viral activity compared to those without HSA.

Effects of HSA: The effects of HSA can be seen more clearly in Figure 7. The "no protein" group comprised a sample size of n=8 formulations and the HSA group comprised a sample size on n=6 formulations. Those formulations with 300mM salt were not included in this comparison. The error bar equals 1 Standard deviation.

As demonstrated above, the addition of HSA significantly increased ($p<0.001$) the plaque number observed in the assay. The reason for this is not known. It appears to be an actual increase in the infectivity of the virus, since it is also observed with a flow cytometer-based assay to determine viral protein expression. Three formulations (#14,16,18) tested increasing HSA concentrations: 1%, 5%, and 10%. These three results are all within the intra-assay variation, so the optimum HSA concentration varies over a broad range.

Effects of Divalent Cations: Samples with less than 300mM NaCl and no added HSA were analyzed for the effect of divalent cations on viral titer after storage at +2-10°C for 14 days. Although there are few replicates, when the samples with divalent cations were compared to those without cations, a non-significant difference was observed. For the limited number of samples tested at +2-10°C (n=4 each group), the addition of divalent cations appeared to result in the loss of virus activity ($p=0.09$; data not shown). This is an unexpected finding, as many literature sources call for the use of divalent cations in adenovirus formulations (Huyghe *et al.*, Human Gene Therapy 6: 1403-1416, 1995).

Comparison to -20°C Reference Formulation Samples: The results of Example 2, in which identical Formulation samples were stored at -20°C for a similar length of time may be used for this comparison. These are compared in Table 7. In general, samples without HSA (Formulations 1-12) showed a loss of viral titers when stored at +4°C compared to storage at -20°C. In contrast, those Formulations with HSA (13-18) showed no loss of viral activity at +4°C. In addition, Formulation samples containing divalent cations (#1,3,5,7,9) had reduced viral titers after storage at +4°C (27% of those kept at -20°C), than those without cations (#2,4,6,8, 10) (66% of those stored at -20°C).

When the adenovirus formulations were stored at +2-10°C for 14 days, the choice of buffer (Tris or PBS) did not have an effect on viral titers, although high NaCl (300mM) concentrations adversely affected virus titer.

Table 7. Comparison of Formulations 1-18 at -20°C and +4°C.

form.	pfu/ml: 4°C	pfu/ml: -20°C	ratio 4°/-20°C
1	2.09×10^9	4.62×10^9	45%
2	1.97×10^9	3.88×10^9	51%
3	1.00×10^9	3.44×10^9	29%
4	2.3×10^9	4.5×10^9	51%
5	4.55×10^8	3.94×10^9	12%
6	1.82×10^9	3.56×10^9	51%
7	6.06×10^8	3.9×10^9	16%
8	2.52×10^9	3.62×10^9	69%
9	1.42×10^9	4.04×10^9	35%
10	5.52×10^9	5.06×10^9	109%
11	9.09×10^7	2.86×10^9	3%
12	6.06×10^7	3.02×10^9	2%
13	9.55×10^9	6.1×10^9	156%
14	9.15×10^9	3.76×10^9	243%
15	7.03×10^9	6.66×10^9	106%
16	6.76×10^9	6.46×10^9	105%
17	8.7×10^9	5.44×10^9	160%
18	6.09×10^9	5.92×10^9	103%

EXAMPLE 4: Ad5CMVp53 Formulation Screening: pH study.

The above Examples with Ad5CMVp53 formulations were all performed at physiological pH, except Formulation 4 shown in Example 1. The experiments within this Example were performed as a short-term study to examine the effects of pH on virus activity over a wide range at -40°C, +20°C, and +37°C. The addition of other excipients was also analyzed for protective effects against extremes of pH. For buffered salt solutions at the extremes of pH 5.0 and pH 9.0, there was a significant loss of virus activity. Viral stability was enhanced at pH values between 6 and about 8, with optimum stability around pH 8.2. In the presence of HSA + sucrose, this effect was negated. In addition, as observed previously, the presence of HSA + sucrose appeared to increase the infectivity of the virus in the plaque assay.

Methods

Virus Sample Preparation: Ad5CMVp53, as described in Examples 1-3 above, was used in these experiments. This virus stock was diluted into formulations with different pH, treated for different storage conditions, and tested for viral titers by the plaque assay as described in Example 1.

Control Virus Sample Preparation: The same starting virus stock was used as in the virus sample preparation above, except that this material was stored in PBS + glycerol at -70°C and thawed on the day of assay.

Formulation Preparation: In the first experiment, a number of 10mM Tris-buffered solutions with the same salt (150mM NaCl) and divalent cation concentrations (2mM $MgCl_2$) were prepared and adjusted to specific pH values, ranging from pH 5.0 to pH 9.0. Another set was made which contained 5% HSA and 5% sucrose in addition to the buffer. For one experiment, virus was diluted into the various formulations, and frozen to -40°C in the rate controlled freezer (length of storage at -40°C varies from 5 to 30 minutes). They were then thawed and incubated in a 37°C water bath for 1 hour prior to activity determination by the plaque assay. In a second experiment, virus was diluted into Formulation 4 (see Table 1), and the pH was adjusted to specific pH values, ranging from pH 6.6 to pH 8.8. These virus formulations were then stored at either room temperature (RT = 20°C) or at 37°C for 1 week prior to activity determination by plaque assay. The viral plaque assays were performed as described in Example 1.

Statistics: Plaque assay data from each of two dilutions and two replicates were combined. Means, standard deviations and CV were calculated. A Student's t-test was performed using Excel 5.0.

Results and Discussion

In the first experiment, two replicates of each pH, ranging from pH 5.0 to pH 9.0, were tested in the plaque assay, following short-term storage at -40°C and 1 hour incubation at 37°C. A second set of formulations with HSA + sucrose added was also tested in this experiment. The standard deviation from the mean was averaged from two dilutions of two replicates. In the first group without HSA + sucrose, the upper and lower extremes of pH resulted in reduced viral titers. The pH 5.0 and pH 9.0 formulation samples were significantly decreased from the mean of the control group: control, pH 6.0, pH 7.0, and pH 8.0 ($p<0.001$ and $p=0.008$, respectively) (data not shown). When HSA + sucrose were present at the various pH levels, no effects of pH were seen (data not shown). In addition, the mean of the group with HSA + sucrose was significantly increased as compared to the mean of the control group without those excipients ($p<0.001$). Thus, the presence of HSA + sucrose protected viral titers from extremes of pH and appeared to enhance the infectivity of the virus in the assay as seen in previous studies (Examples 1-3). Therefore, for buffered salt formulations, the optimum pH range for Ad5CMVp53 is pH 6.0-pH 8.0.

The addition of HSA + sucrose is able to protect the virus from pH values above and below this range.

In a second experiment, virus was prepared in Formulation 4 at each specific pH value, ranging from pH 6.6 to pH 8.8, stored for 1 week at room temperature or 37°C, and tested in the plaque assay. The results are shown in Figure 8.

In general, the formulations with pH from 8.0 to 8.6 maintained the highest level of activity following storage at room temperature for one week, with the virus sample at pH 8.2 retaining the most activity (Figure 8). The "too numerous to count" (tntc) data designation indicates that the plaque counts for these samples were above the upper limit for counting. Therefore, a value of 300 plaques, which is the limit for counting, was assigned to these "tntc" samples and used to calculate the titer reported in Figure 8. The real titer is slightly higher than this assigned value. Following storage at 37°C for one week, Formulation 4 having a pH value of pH 8.0, pH 8.4, and pH 8.6 resulted in the best preservation of virus stability (Figure 8). Taken together, the stability data obtained from the room temperature and 37°C short term pH study indicates that Formulation 4, with an adjusted pH value, ranging from pH 8.0 to pH 8.6 is the most preferable formulation to preserve virus activity (infectivity).

EXAMPLE 5: Bovine serum albumin and recombinant human albumin provide similar effect as natural HSA.

The purpose of the studies within this Example was to determine whether albumin from other sources can provide the same stability and viral titer enhancement observed with HSA in Example 2. These studies were also performed to investigate the possibility that the other components in the commercial HSA (0.02M acetyltryptophane and 0.02M sodium caprylate; Miles, Inc.) contribute to stability and titer enhancement in HSA-based viral formulations. In these studies bovine serum albumin (BSA, reagent grade material, powder form) and recombinant human albumin (rHA) were tested in different studies under different conditions. The recombinant human albumin used is Recombumin™ 25 which is produced in a yeast expression system by Centeon and is described *supra*.

Methods

Virus Sample Preparation: Ad5CMVp53, as used in other Examples, was also used for these experiments. For both the BSA and the Recombumin™ studies, the stock virus was diluted 1:100 into the test formulations, stored at various temperatures for different lengths of time, and were tested for viral titer (infectivity) using the plaque assay as described in Example 1.

Formulation Preparation: In the BSA study, three viral formulations were prepared: Control Formulation: DPBS + 10% glycerol + 0.5mM $MgCl_2$ + 1.0mM $CaCl_2$; HSA Formulation: DPBS + 5% HSA + 0.5mM $MgCl_2$ + 1.0mM $CaCl_2$; and BSA Formulation: DPBS + 5% BSA + 0.5mM $MgCl_2$ +

1.0mM $CaCl_2$. The BSA used in this study was a reagent grade material and in powder form. The virus stock was diluted 1:100 into each formulation and divided into two vials each. One vial was stored at RT (20°C) for 2 hours and the other vial was frozen to -40°C in a rate controlled freezer then thawed and stored at +37°C for 2 hours. Viral plaque testing was performed as described in Example 1.

In the Recombumin™ study, three formulations were prepared: Control Formulation: DPBS + 10% glycerol; HSA Formulation: 10mM Tris + 5% HSA (w/v) + 5% sucrose + 150 mM NaCl + 2.0mM $MgCl_2$, pH 8.2; and Recombumin™ Formulation: 10mM Tris + 5% Recombumin™ (w/v) + 5% sucrose + 150 mM NaCl + 2.0mM $MgCl_2$, pH 8.2. The virus stock was diluted 1:100 into each formulation and divided into 3 vials each and stored at +37°C for 7 days. Viral plaque testing was performed as described in Example 1.

Results and Discussion

The results of the BSA study showed that the BSA Formulation enhanced viral titer, as observed for the HSA Formulation, when compared to the viral titer of the Control Formulation (Figure 9). In addition, similar results were observed when murine serum albumin was used in the storage formulation instead of BSA or HSA (data not shown). The data also indicated that HSA, BSA, and the Control Formulations appear to have a protective effect on the virus undergoing a freeze-thaw and up to 2 hour storage at +37°C (Figure 9).

The results of the Recombumin™ study demonstrated that the Recombumin™ and HSA Formulations provide similar protective effects on viral titer for viruses stored at +37°C for 1 week (Figure 10). This protective effect was not observed with the Control Formulation in which a viral titer decrease of 4 logs was observed (Figure 10).

The results of this Example demonstrate that formulations containing albumin from other sources (bovine and recombinant human) can provide the same stability and viral titer enhancement as that observed with the natural HSA Formulation. These studies also indicate that the other components in the commercial HSA (0.02M acetyltryptophane and 0.02M sodium caprylate) do not contribute to the stability and titer enhancement in HSA-based viral formulations.

EXAMPLE 6: Long Term Stability of Adenovirus in an HSA/Sucrose Formulation at -70°C, -20°C, +4°C, and +20°C.

The results of Examples 1-5 presented *supra* indicate that a liquid formulation containing human serum albumin (HSA) provides superior stabilization for adenoviral vectors compared to those containing other excipients. The purpose of this Example was to determine the stability of adenoviral vectors in a 10mM Tris-HCl + 5% HSA + 5% sucrose + 150mM NaCl + 2mM $MgCl_2$, pH 8.4

formulation when stored at various temperatures up to 8.5 months. An adenoviral vector comprising the herpes simplex virus thymidine kinase gene (AV1.0HSVTK) was used in this Example.

This Example summarizes the efficiency of a formulation comprising 10mM Tris-HCl + 5% HSA + 5% sucrose + 150mM NaCl + 2mM $MgCl_2$, pH 8.4 (Formulation 19) to preserve adenoviral vectors at four storage temperatures for 0 (2 days), 1.5, 3.5, and 8.5 months. This study was designed to determine optimal formulation of an adenovirus vector to achieve at least one (1) year stability when stored frozen at -20°C, or as a liquid at +4°C or +20°C storage temperatures.

Methods

Virus Sample Preparation: A type 5 adenovirus comprising the HSV-TK gene under the control of the cytomegalovirus promoter, AV1.0HSVTK (as described in French Patent Application No. FR93/13772), in DPBS + 10% glycerol, 0.2 μm filtered, was used in these experiments. The virus titer was estimated at 1.6×10^{12} particles/ml. On the first day of the study, 22 vials of AV1.0HSVTK, each vial comprising about 200-250 μl/vial for a total of 4.5 ml, were thawed at room temperature and pooled. OD measurements were taken at 260nm on the pooled material to determine the original particles/ml.

The AV1.0HSVTK virus pooled stocks were run through a Resource Q column (prepared with 8ml of Source Q15; Pharmacia Catalog No. 17-0947-01, lot 11/21/97 for buffer exchange and reformulated in 10mM Tris-HCl, 5% HSA, 5% sucrose, 150mM NaCl, 2mM $MgCl_2$, pH 8.4 (Formulation 19). All solutions used in this buffer exchange process were degassed by an in-line degasser and the virus solution was purged with argon prior to filling. The material was aliquoted into polypropylene cryovials and flushed with argon to remove oxygen from the head space of vials. The samples were then placed at -20°C (VWR Scientific freezer), +4°C (VWR Scientific 2-door deli case Model GDM-49), room temperature (+20°C), and -70°C (control group). Triplicate vials were tested for particle integrity by HPLC and activity by plaque assay at specified time points of up to 8.5 months. After the final dilution and filling, all samples were stored at each desired temperature for 2 days and then tested as T=0 time point to give a baseline information. The samples for -20°C storage were frozen to -40°C in a controlled rate freezer prior to storage at -20°C. The temperature of +20°C was used as an elevated temperature with which to determine the "accelerated" stability of the virus formulation for +4°C. Triplicate virus samples and positive controls were tested at intervals of 0 (2 days), 1.5, 3.5, and 8.5 months at -20°C, +4°C, and +20°C with respect to viral particle integrity and viral infectivity. Viral titer in plaque forming units (pfu) was determined by plaque assay using 293 cells. An analytical HPLC method was used to determine viral particle/ml measurements by peak area of an optical density (OD) at 260nm.

Positive Control Virus Preparation: The control virus was diluted in 10mM Tris-HCl, 5% HSA, 5% sucrose, 150mM NaCl, 2mM $MgCl_2$, pH 8.4 (Formulation 19) to mimic the test sample formulation and was aliquoted, 0.4 ml per cryovial (Nalgene No. 5000-0020, lot 072381), and stored at -70°C (BioFreezer, Forma Scientific Model No. 8328). Triplicate virus samples and positive controls were tested at intervals of 0 (2 days), 1.5, 3.5, and 8.5 months at -70°C with respect to viral particle integrity and viral infectivity. Viral titer in plaque forming units (pfu) was determined by plaque assay using 293 cells. An analytical HPLC method was used to determine viral particle/ml measurements by peak area of an optical density (OD) at 260nm.

Plaque Assay Analysis: Viral titer was determined by the plaque assay on 293 cells, human embryonic kidney cells transformed with sheared human Ad5 DNA (complement E1 region). At designated time points, triplicate test samples and triplicate positive controls were tested for plaque forming units as described above in Example 1.

HPLC Analysis: HPLC peak area comparisons were performed to determine the virus concentration in terms of viral particles between samples and positive controls. Each triplicate sample and positive control was tested on a Waters analytical HPLC system with Millennium software as described above in Example 1.

Results and Discussion

Plaque Assay Results: The results of the plaque assays for adenovirus AV1.0HSVTK stability at -70°C, -20°C, +4°C, and +20°C are shown in Figure 11. The viral activity of AV1.0HSVTK was measured after 0 (2 days), 1.5, 3.5, and 8.5 months of storage using the plaque forming assay as described in Example 1. Both the +4°C and -20°C storage temperature samples maintained similar viral activities as compared to the -70°C positive control at all tested time points (Figure 11). The results showed a significant drop ($p=0.02$) of approximately 2 logs for the room temperature condition. All other storage conditions preserved viral activity to an amount within the error limit of the assay (±0.5 logs).

HPLC Results: HPLC peak area provides for the quantitation of viral particles. The HPLC assay variation has been determined to be ± 3%. Due to the small standard deviations in the HPLC peak areas, any small variation between time points may be statistically significant. The HPLC peak area was plotted by temperature over time for all four storage temperatures. Analysis by HPLC of particle integrity was performed following storage of the test and control samples for 0 (2 days), 1.5, 3.5, and 8.5 months. The results demonstrate that all storage conditions of -20°C, +4°C, and +20°C preserved viral particles as well as the -70°C condition over the course of 8.5 months (data not shown).

In summary, the HPLC results showed a similar trend in stability as the plaque assay results for

each storage temperature. Formulation 19 preserved adenoviral titer for at least 8.5 months when stored at -70°C, -20°C, +4°C, or +20°C. Interestingly, the HPLC result did not suggest a loss of activity in the room temperature condition at the 8.5 month time point as demonstrated by the plaque assay results (Figure 11).

The results of this study clearly demonstrate that Formulation 19 (10mM Tris-HCL, pH 8.4, 5% HSA, 5% sucrose, 2.0mM $MgCl_2$, and 150mM NaCl) is effective for stably preserving viral activity and particle integrity at all four storage temperatures analyzed over an 8.5 month time period. The plaque assay (Figure 11) and HPLC data support this conclusion. The results from both particle integrity tests and activity titration show that viral stability is preserved up to at least 8.5 months under frozen (-20°C) and (+4°C) refrigerated conditions. However, viral activity, but not particle integrity, was reduced when storage was performed in Formulation 19 at room temperature. Therefore, Formulation 19 comprising 5% human serum albumin and 5% sucrose at a pH of 8.4 efficiently preserves and stabilizes adenoviral particle integrity and viral activity for a period of at least 8.5 months at refrigerated conditions.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A composition comprising a recombinant adenovirus vector and a concentration of human serum albumin (HSA) effective to stabilize the adenovirus vector at a temperature above the freezing point of water or to enhance a titer of the adenovirus vector compared to a titer in the absence of HSA, or both, in an aqueous buffer.

2. The composition of claim 1, wherein the concentration of HSA is from about 0.01% to about 25% (w/v).

3. The composition of claim 2, wherein the concentration of HSA is from about 0.1% to about 15%.

4. The composition of claim 3, wherein the concentration of HSA is from about 1% to about 10%.

5. The composition of claim 4, wherein the concentration of HSA is about 5%.

6. The composition of claim 1, wherein the pH is greater than or equal to 5.0 and less than or equal to 9.0.

7. The composition of claim 6, wherein the pH is greater than 7.5.

8. The composition of claim 7, wherein the pH is greater than 8.0.

9. The composition of claim 8, wherein the pH is 8.2.

10. The composition of claim 8, wherein the pH is 8.4.

11. The composition of claim 4, wherein the pH is greater than 8.0.

12. The composition of claim 5, wherein the pH is 8.2.

13. The composition of claim 5, wherein the pH is 8.4.

14. The composition of claim 1, wherein the buffer is a Tris-HCl buffer.

15. The composition of claim 11, wherein the buffer is a Tris-HCl buffer.

16. The composition of claim 12, wherein the buffer is a Tris-HCl buffer.

17. The composition of claim 13, wherein the buffer is a Tris-HCl buffer.

18. The composition of claim 1, further comprising about 5% sucrose, about 2.0 mM $MgCl_2$ and about 150 mM NaCl.

19. The composition of claim 15, further comprising about 5% sucrose, about 2.0 mM $MgCl_2$ and about 150 mM NaCl.

20. The composition of claim 16, further comprising about 5% sucrose, about 2.0 mM $MgCl_2$ and 150 mM NaCl.

21. The composition of claim 17, further comprising about 5% sucrose, about 2.0 mM $MgCl_2$ and 150 mM NaCl.

22. The composition of claim 1, wherein the recombinant adenovirus expresses a heterologous protein.

23. The composition of claim 22, wherein the heterologous protein is p53.

24. The composition of claim 22, wherein the heterologous protein is HSV-TK.

25. A method for preparing a stabilized recombinant adenovirus formulation comprising suspending a recombinant adenovirus in an aqueous buffer comprising a concentration of human serum albumin (HSA) effective to stabilize the adenovirus vector at a temperature above the freezing point of water, or enhance a titer of the adenovirus vector compared to a titer in the absence of HSA.

26. The method according to claim 25, wherein the temperature is greater than or equal to 4°C and less than 37°C.

27. The method according to claim 25, wherein the temperature is greater than or equal to 20°C.

28. The method according to claim 26, wherein the concentration of HSA is 5%.

29. The method according to claim 26, wherein the pH of the admixture is greater than 8.0.

30. The method according to claim 26, wherein the pH of the admixture is 8.2.

31. The method according to claim 26, wherein the pH of the admixture is 8.4.

32. A method for stabilizing an adenovirus vector at about 20°C, which comprises preparing an

admixture of the adenovirus vector in an aqueous composition of Dulbecco's phosphate buffered saline, from about 5% to 15% glycerol, from about 0.25 to 2.0 mM $CaCl_2$, and from about 0.1 to 1.0 mM $MgCl_2$.

33. The method according to claim 32, wherein the concentration of glycerol is about 10%, the concentration of $CaCl_2$ is about 1.0 mM, and the concentration of $MgCl_2$ is about 0.5 mM.

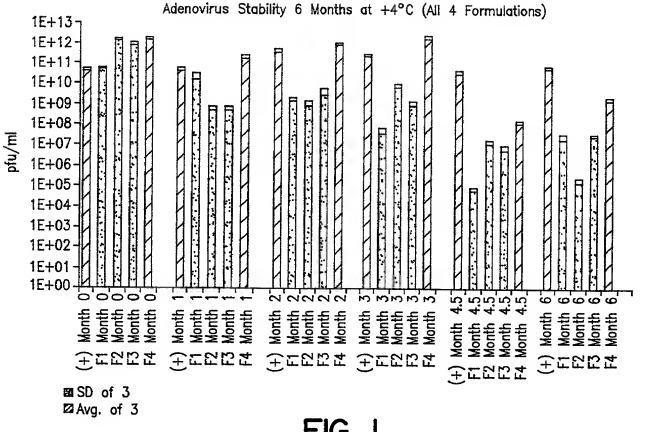

Adenovirus Stability 6 Months at +4°C (All 4 Formulations)
pfu/ml
1E+13
1E+12
1E+11
1E+10
1E+09
1E+08
1E+07
1E+06
1E+05
1E+04
1E+03
1E+02
1E+01
1E+00
(+) Month 0
F1 Month 0
F2 Month 0
F3 Month 0
F4 Month 0
(+) Month 1
F1 Month 1
F2 Month 1
F3 Month 1
F4 Month 1
(+) Month 2
F1 Month 2
F2 Month 2
F3 Month 2
F4 Month 2
(+) Month 3
F1 Month 3
F2 Month 3
F3 Month 3
F4 Month 3
(+) Month 4.5
F1 Month 4.5
F2 Month 4.5
F3 Month 4.5
F4 Month 4.5
(+) Month 6
F1 Month 6
F2 Month 6
F3 Month 6
F4 Month 6
SD of 3
Avg. of 3

FIG. 1

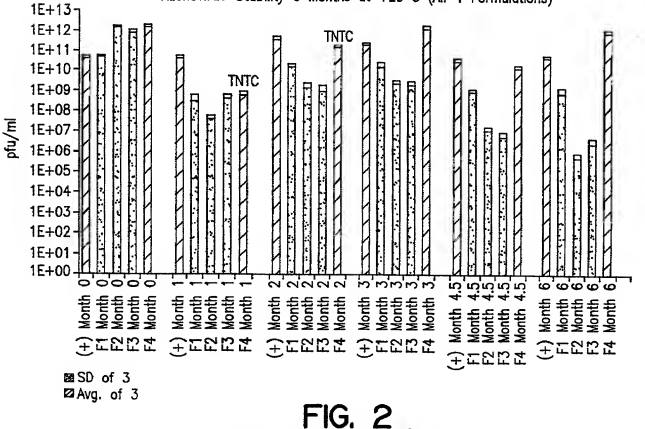

Adenovirus Stability 6 Months at +20°C (All 4 Formulations)
TNTC
TNTC
pfu/ml
1E+13
1E+12
1E+11
1E+10
1E+09
1E+08
1E+07
1E+06
1E+05
1E+04
1E+03
1E+02
1E+01
1E+00
(+) Month 0
F1 Month 0
F2 Month 0
F3 Month 0
F4 Month 0
(+) Month 1
F1 Month 1
F2 Month 1
F3 Month 1
F4 Month 1
(+) Month 2
F1 Month 2
F2 Month 2
F3 Month 2
F4 Month 2
(+) Month 3
F1 Month 3
F2 Month 3
F3 Month 3
F4 Month 3
(+) Month 4.5
F1 Month 4.5
F2 Month 4.5
F3 Month 4.5
F4 Month 4.5
(+) Month 6
F1 Month 6
F2 Month 6
F3 Month 6
F4 Month 6
SD of 3
Avg. of 3

FIG. 2

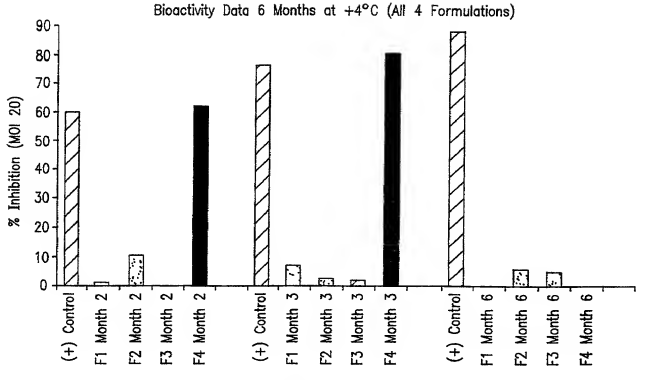

Bioactivity Data 6 Months at +4°C (All 4 Formulations)
% Inhibition (MOI 20)
90
80
70
60
50
40
30
20
10
0
(+) Control
F1 Month 2
F2 Month 2
F3 Month 2
F4 Month 2
(+) Control
F1 Month 3
F2 Month 3
F3 Month 3
F4 Month 3
(+) Control
F1 Month 6
F2 Month 6
F3 Month 6
F4 Month 6

FIG. 3

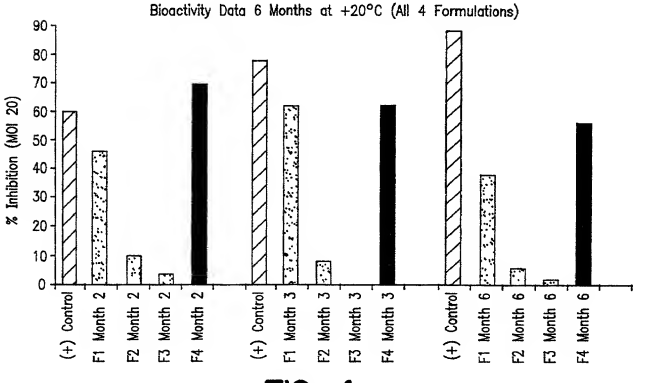
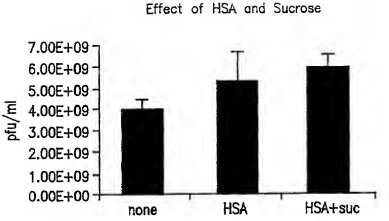
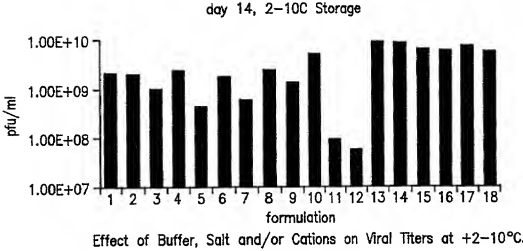

Bioactivity Data 6 Months at +20°C (All 4 Formulations)
% Inhibition (MOI 20)
90
80
70
60
50
40
30
20
10
0
(+) Control
F1 Month 2
F2 Month 2
F3 Month 2
F4 Month 2
(+) Control
F1 Month 3
F2 Month 3
F3 Month 3
F4 Month 3
(+) Control
F1 Month 6
F2 Month 6
F3 Month 6
F4 Month 6

FIG. 4



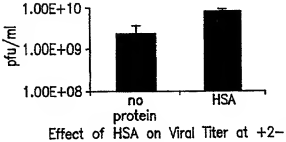
Effect of HSA and Sucrose on Viral Titers at −20 °C.

FIG. 5



Effect of Buffer, Salt and/or Cations on Viral Titers at +2−10°C.

FIG. 6



Effect of HSA on Viral Titer at +2−10 °C.

FIG. 7

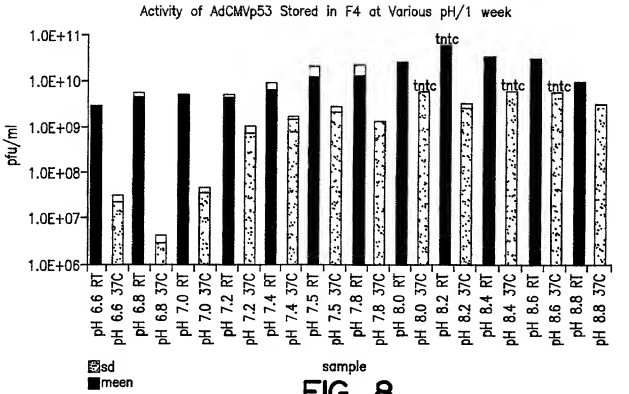

Activity of AdCMVp53 Stored in F4 at Various pH/1 week
1.0E+11
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
pfu/ml
tntc
pH 6.6 RT
pH 6.6 37C
pH 6.8 RT
pH 6.8 37C
pH 7.0 RT
pH 7.0 37C
pH 7.2 RT
pH 7.2 37C
pH 7.4 RT
pH 7.4 37C
pH 7.5 RT
pH 7.5 37C
pH 7.8 RT
pH 7.8 37C
pH 8.0 RT
pH 8.0 37C
pH 8.2 RT
pH 8.2 37C
pH 8.4 RT
pH 8.4 37C
pH 8.6 RT
pH 8.6 37C
pH 8.8 RT
pH 8.8 37C
sd
meen
sample

FIG. 8

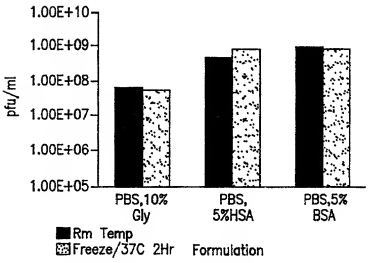

Comparsion of HSA and BSA in viral formulations
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
pfu/ml
PBS,10% Gly
PBS, 5%HSA
PBS,5% BSA
Rm Temp
Freeze/37C 2Hr
Formulation

FIG. 9

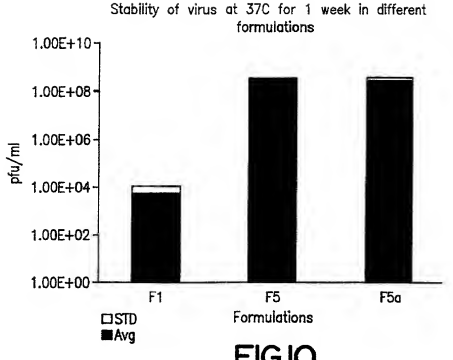


FIG.10

F1: DPBS + 10% glycerol
F5: 10mM Tris + 5% HSA + 5% sucrose + 150 mM NaCl + 2.0mM $MgCl_2$, pH 8.2
F5a: 10mM Tris + 5% Recombumin + sucrose + 150 mM NaCl + 2.0mM $MgCl_2$, pH 8.2

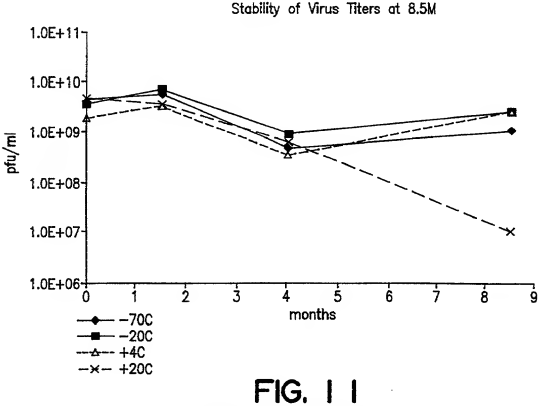


FIG. 11

UNITED STATES OF AMERICA

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor(s), I/We hereby declare that:

My/Our residence(s), post office address(es) and citizenship(s) are as stated below my name(s).
I/We verily believe I am/we are the original, first and sole/joint inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Adenovirus Formulations for Gene Therapy

and the specification of which (check one)
- ☐ is attached hereto (Attorney Docket No.)
- ☐ was filed on as U.S. Application Number and was amended on (if applicable).
- ☒ was described and claimed in PCT Int'l Application Number **PCT/US99/18515** filed on **13 August 1999** and as amended under PCT Article 19 on (if any).

I/We hereby state that I/We have reviewed and understand the contents of the above identified specification, including the claims as amended by any amendment referred to above.
I/We acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 C.F.R. 1.56.

☐ I/We hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or 365 (b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one other country other than the United States of America, listed below and having a filing date before that of the application on which priority is claimed. I/We have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

	Number	Country	Day/Month/Year Filed
Foreign Priority:			
Prior Foreign Appln(s):			

☒ I/We hereby claim the benefit under Title 35, United States Code §119(e) of any United States Provisional application(s) listed below:

60/096,600	14 August 1998
Number	Filing Date

☒ I/We hereby claim the benefit under Title 35, United States Code §120 or 365(c) of any United States application(s) or international application designating the United States listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I/We acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

PCT/US99/18515	13 August 1999	Pending
Application Serial No.	Filing Date	Status (Patented, Pending)

I/We hereby appoint the attorneys and/or agents associated with the Customer No.(s) provided below as my/our attorneys and/or agents with full power to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Customer No. 005487

I/We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States code §1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Inventors(s): ☐ Additional names and signatures are attached.

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Signature: Dewey J. Moody
Date: June 25, 2001
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Signature: _____
Date: _____
Country of Citizenship: _____
Residence: _____
(City and State/City and Country only)
P. O. Address: _____

5. Full name: _____

Signature: _____
Date: _____
Country of Citizenship: _____
Residence: _____
(City and State/City and Country only)
P. O. Address: _____

6. Full name: _____

Signature: _____
Date: _____
Country of Citizenship: _____
Residence: _____
(City and State/City and Country only)
P. O. Address: _____

7. Full name: _____

Signature: _____
Date: _____
Country of Citizenship: _____
Residence: _____
(City and State/City and Country only)
P. O. Address: _____

8. Full name: _____

Signature: _____
Date: _____
Country of Citizenship: _____
Residence: _____
(City and State/City and Country only)
P. O. Address: _____

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Telefax (908) 231-2626

Inventors(s): ☐ Additional names and signatures are attached.

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Foster City, CA 94404

2. Full name: **Karen R. MCGLENNON**

Signature: _____

Date: _____

Country of Citizenship: **US**

Residence: **Sunnyvale, CA**
(City and State/City and Country only)

P. O. Address: **827 Lakewood Drive**
Sunnyvale, CA 94089

3. Full name: **Dewey MOODY**

Signature: _____

Date: _____

Country of Citizenship: **US**

Residence: **Seattle, WA**
(City and State/City and Country only)

P. O. Address: **2520 26th Avenue West**
Seattle, WA 98119

4. Full name: _____

Signature: _____

Date: _____

Country of Citizenship: _____

Residence: _____
(City and State/City and Country only)

P. O. Address: _____

5. Full name: _____

Signature: _____

Date: _____

Country of Citizenship: _____

Residence: _____
(City and State/City and Country only)

P. O. Address: _____

6. Full name: _____

Signature: _____

Date: _____

Country of Citizenship: _____

Residence: _____
(City and State/City and Country only)

P. O. Address: _____

7. Full name: _____

Signature: _____

Date: _____

Country of Citizenship: _____

Residence: _____
(City and State/City and Country only)

P. O. Address: _____

8. Full name: _____

Signature: _____

Date: _____

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Residence: _____
(City and State/City and Country only)

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6. Full name: _____

Signature: _____
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Date: _____
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8. Full name: _____

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UNITED STATES OF AMERICA

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor(s), I/We hereby declare that:

My/Our residence(s), post office address(es) and citizenship(s) are as stated below my name(s).
I/We verily believe I am/we are the original, first and sole/joint inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Adenovirus Formulations for Gene Therapy

and the specification of which (check one)
- ☐ is attached hereto (Attorney Docket No.)
- ☐ was filed on as U.S. Application Number and was amended on (if applicable).
- ☒ was described and claimed in PCT Int'l Application Number **PCT/US99/18515**

filed on **13 August 1999** and as amended under PCT Article 19 on (if any).

I/We hereby state that I/We have reviewed and understand the contents of the above identified specification, including the claims as amended by any amendment referred to above.
I/We acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 C.F.R. 1.56.

☐ I/We hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or 365 (b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one other country other than the United States of America, listed below and having a filing date before that of the application on which priority is claimed. I/We have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

	Number	Country	Day/Month/Year Filed
Foreign Priority:			
Prior Foreign Appln(s):			

☒ I/We hereby claim the benefit under Title 35, United States Code §119(e) of any United States Provisional application(s) listed below:

Number	Filing Date
60/096,600	14 August 1998

☒ I/We hereby claim the benefit under Title 35, United States Code §120 or 365(c) of any United States application(s) or international application designating the United States listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I/We acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status (Patented, Pending)
PCT/US99/18515	13 August 1999	Pending

I/We hereby appoint the attorneys and/or agents associated with the Customer No.(s) provided below as my/our attorneys and/or agents with full power to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Customer No.: 005487

I/We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States code §1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Inventor(s): ☐ Additional names and signatures are attached.

1. Full name: **Shian-Jiun SHIH**

2. Full name: **Karen R. MCGLENNON**

UNITED STATES OF AMERICA

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I/We verily believe I am/we are the original, first and sole/joint inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Adenovirus Formulations for Gene Therapy

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	Number	Country	Day/Month/Year Filed
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Prior Foreign Appln(s):			

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PCT/US99/18515	13 August 1999	Pending

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APPLICATION INFORMATION

Title Line One:: Adenovirus Formulations For Gene Therapy
Formal Drawings?:: No
Application Type:: Utility
Docket Number:: A2922A US
Secrecy Order in Parent Appl.?:: No

CONTINUITY INFORMATION

This application is a:: NON PROV. OF PROVISIONAL
> Application One:: 60/096,600
Filing Date:: 08-14-1998

PRIOR FOREIGN APPLICATIONS

Foreign Application One:: PCT/US99/18515
Filing Date:: 08-13-1998
Country:: PCT
Priority Claimed:: Yes

Source:: PrintEFS Version 1.0.1